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(54) Title: GENE SEQUENCE VARIANCES WITH UTILITY IN DETERMINING THE TREATMENT OF DISEASE

(57) Abstract

The present disclosure describes the use of genetic variance information for folate transport or metabolism genes or pyrimidine transport or metabolism genes in the selection of effective methods of treatment of a disease or condition. The variance information is indicative of the expected response of a patient to a method of treatment. Methods of determining relevant variance information and additional methods of using such variance information are also described.

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DESCRIPTION

GENE SEQUENCE VARIANCES WITH
UTILITY IN DETERMINING THE TREATMENT OF DISEASE

RELATED APPLICATIONS

This application is a CIP of Stanton, U.S. Patent Application Serial No. not yet assigned, attorney docket 244/273, filed July 19, 1999, entitled GENE SEQUENCE VARIANCES WITH UTILITY IN DETERMINING THE TREATMENT OF DISEASE and claims the benefit of Stanton, U.S. Provisional Application 60/093,484, filed July 20, 1998, entitled GENE SEQUENCE VARIANCES WITH UTILITY IN DETERMINING THE TREATMENT OF DISEASE, which are hereby incorporated by reference in their entireties including drawings and tables.

BACKGROUND OF THE INVENTION

This application concerns the field of mammalian therapeutics and the selection of therapeutic regimens utilizing host genetic information, including gene sequence variances within the human genome in human populations.

The rate of approval of new drugs that enter human clinical trials is less than 20%, despite demonstrated efficacy of said new drugs in preclinical models of human disease. In some instances the low response rate in humans is due to genetic heterogeneity in the drug target or the pathway mediating the action of the drug. Identification of the genetic causes of variable drug response would allow more rational clinical development of drugs. Further, many drugs or other treatments approved for use in humans are known to have highly variable safety and efficacy in different individuals. A consequence of such variability is that a given drug or other treatment may be highly effective in one individual, and ineffective or not well tolerated in another individual. Thus, administration of such a drug to an individual in whom the drug would be ineffective would result in wasted cost and time during which the patient's condition may significantly worsen. Also, administration of a drug to an individual in whom the drug would not be tolerated could result in a direct worsening of the patient's condition and could even result in the patient's death.

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For some drugs, up to 99% of the measurable variation in selected pharmacokinetic parameters has been shown to be inherited, or associated with genetic factors. Studies have also demonstrated a significant genetic component to pharmacodynamic variation. For a limited number of drugs, discrete gene sequence

5   variances have been identified in specific genes that are involved in drug action, and these variances have been shown to account for the variable efficacy or safety of the drug in different individuals.

SUMMARY OF THE INVENTION

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The present invention is concerned generally with the field of treatment of diseases and conditions in mammals, particularly in humans. It is concerned with the genetic basis of inter-patient variation in response to therapy, including drug therapy. Specifically, this invention describes the identification of gene sequence variances useful

15   in the field of therapeutics for optimizing efficacy and safety of drug therapy for specific diseases or conditions and for establishing diagnostic tests useful for improving the development and use of pharmaceutical products in the clinic. Methods for identifying genetic variances and determining their utility in the selection of optimal therapy for specific patients are also described, along with probes and related materials which are

20   useful, for example, in identifying the presence of a particular gene sequence variance in cells of an individual. The genes involved in the present invention are those listed in a pathway, gene table, list or example herein.

The inventors have determined that the identification of gene sequence variances within genes that may be involved in drug action is important for determining whether

25   genetic variances account for variable drug efficacy and safety and for determining whether a given drug or other therapy may be safe and effective in an individual patient. Provided in this invention are identifications of genes and sequence variances which can be useful in connection with predicting differences in response to treatment and selection of appropriate treatment of a disease or condition. Such genes and variances have utility

30   in pharmacogenetic association studies and diagnostic tests to improve the use of certain drugs or other therapies including, but not limited to, the drug classes and specific drugs identified in the 1999 Physicians' Desk Reference (53rd edition), Medical Economics

Data, 1998, or the 1995 United States Pharmacopeia XXIII National Formulary XVIII, Interpharm Press, 1994, or other sources as described below.

The terms "disease" or "condition" are commonly recognized in the art and designate the presence of signs and/or symptoms in an individual or patient that are

5   generally recognized as abnormal. Diseases or conditions may be diagnosed and categorized based on pathological changes. Signs may include any objective evidence of a disease such as changes that are evident by physical examination of a patient or the results of diagnostic tests which may include, among others, laboratory tests to determine the presence of variances or variant forms of certain genes in a patient. Symptoms are

10   subjective evidence of disease or a patients condition – i.e. the patients perception of an abnormal condition that differs from normal function, sensation, or appearance, which may include, without limitations, physical disabilities, morbidity, pain, and other changes from the normal condition experienced by an individual. Various diseases or conditions include, but are not limited to, those categorized in standard textbooks of medicine

15   including, without limitation, textbooks of nutrition, allopathic, homeopathic, and osteopathic medicine. In certain aspects of this invention, the disease or condition is selected from the group consisting of the types of diseases listed in standard texts such as Harrison's Principles of Internal Medicine (14th Ed) by Anthony S. Fauci, Eugene Braunwald, Kurt J. Isselbacher, et al. (Editors), McGraw Hill, 1997, or Robbins

20   Pathologic Basis of Disease (6th edition) by Ramzi S. Cotran, Vinay Kumar, Tucker Collins & Stanley L. Robbins, W B Saunders Co., 1998, or the Diagnostic and Statistical Manual of Mental Disorders: Dsm-IV (4th Ed), American Psychiatric Press, 1994 or other texts described below.

In connection with the methods of this invention, unless otherwise indicated, the

25   term "suffering from a disease or condition" means that a person is either presently subject to the signs and symptoms, or is more likely to develop such signs and symptoms than a normal person in the population. Thus, for example, a person suffering from a condition can include a developing fetus, a person subject to a treatment or environmental condition which enhances the likelihood of developing the signs or symptoms of a

30   condition, or a person who is being given or will be given a treatment which increase the likelihood of the person developing a particular condition. For example, tardive dyskinesia is associated with long-term use of anti-psychotics; gastrointestinal symptoms, alopecia and bone marrow suppression are associated with cancer chemotherapeutic

regimens, and immunosuppression is associated with agents to limit graft rejection following transplantation. Thus, methods of the present invention which relate to treatments of patients (e.g., methods for selecting a treatment, selecting a patient for a treatment, and methods of treating a disease or condition in a patient) can include primary treatments directed to a presently active disease or condition, secondary treatments which are intended to cause a biological effect relevant to a primary treatment, and prophylactic treatments intended to delay, reduce, or prevent the development of a disease or condition, as well as treatments intended to cause the development of a condition different from that which would have been likely to develop in the absence of the treatment.

The term "therapy" refers to a process which is intended to produce a beneficial change in the condition of a mammal, e.g., a human, often referred to as a patient. A beneficial change can, for example, include one or more of: restoration of function, reduction of symptoms, limitation or retardation of progression of a disease, disorder, or condition or prevention, limitation or retardation of deterioration of a patient's condition, disease or disorder. Such therapy can involve, for example, nutritional modifications, administration of radiation, administration of a drug, behavioral modifications and combinations of these, among others.

The term "drug" as used herein refers to a chemical entity or biological product, or combination of chemical entities or biological products, administered to a person to treat or prevent or control a disease or condition. The chemical entity or biological product is preferably, but not necessarily a low molecular weight compound, but may also be a larger compound, for example, an oligomer of nucleic acids, amino acids, or carbohydrates including without limitation proteins, oligonucleotides, ribozymes, DNAzymes, glycoproteins, lipoproteins, and modifications and combinations thereof. A biological product is preferably a monoclonal or polyclonal antibody or fragment thereof such as a variable chain fragment cells; or an agent or product arising from recombinant technology, such as, without limitation, a recombinant protein, recombinant vaccine, or DNA construct developed for therapeutic, e.g., human therapeutic, use. The term "drug" may include, without limitation, compounds that are approved for sale as pharmaceutical products by government regulatory agencies (e.g., U.S. Food and Drug Administration (USFDA or FDA), European Medicines Evaluation Agency (EMEA), and a world regulatory body governing the Internation Conference of Harmonization (ICH) rules and

guidelines), compounds that do not require approval by government regulatory agencies, food additives or supplements including compounds commonly characterized as vitamins, natural products, and completely or incompletely characterized mixtures of chemical entities including natural compounds or purified or partially purified natural

5   products. The term "drug" as used herein is synonymous with the terms "medicine", "pharmaceutical product", or "product". Most preferably the drug is approved by a government agency for treatment of a specific disease or condition.

A "low molecular weight compound" has a molecular weight <5,000 Da, more preferably <2500 Da, still more preferably <1000 Da, and most preferably <700 Da.

10   Those familiar with drug use in medical practice will recognize that regulatory approval for drug use is commonly limited to approved indications, such as to those patients afflicted with a disease or condition for which the drug has been shown to be likely to produce a beneficial effect in a controlled clinical trial. Unfortunately, it has generally not been possible with current knowledge to predict which patients will have a

15   beneficial response, with the exception of certain diseases such as bacterial infections where suitable laboratory methods have been developed. Likewise, it has generally not been possible to determine in advance whether a drug will be safe in a given patient. Regulatory approval for the use of most drugs is limited to the treatment of selected diseases and conditions. The descriptions of approved drug usage, including the

20   suggested diagnostic studies or monitoring studies, and the allowable parameters of such studies, are commonly described in the "label" or "insert" which is distributed with the drug. Such labels or inserts are preferably required by government agencies as a condition for marketing the drug and are listed in common references such as the Physicians Desk Reference (PDR). These and other limitations or considerations on the

25   use of a drug are also found in medical journals, publications such as pharmacology, pharmacy or medical textbooks including, without limitation, textbooks of nutrition, allopathic, homeopathic, and osteopathic medicine.

Many widely used drugs are effective in a minority of patients receiving the drug, particularly when one controls for the placebo effect. For example, the PDR shows that

30   about 45% of patients receiving Cognex (tacrine hydrochloride) for Alzheimer's disease show no change or minimal worsening of their disease, as do about 68% of controls (including about 5% of controls who were much worse). About 58% of Alzheimer's patients receiving Cognex were minimally improved, compared to about 33% of controls,

while about 2% of patients receiving Cognex were much improved compared to about 1% of controls. Thus a tiny fraction of patients had a significant benefit. Response to many cancer chemotherapy drugs is even worse. For example, 5-fluorouracil is standard therapy for advanced colorectal cancer, but only about 20-40% of patients have an objective response to the drug, and, of these, only 1-5% of patients have a complete response (complete tumor disappearance; the remaining patients have only partial tumor shrinkage). Conversely, up to 20-30% of patients receiving 5-FU suffer serious gastrointestinal or hematopoietic toxicity, depending on the regimen.

Thus, in a first aspect, the invention provides a method for selecting a treatment for a patient suffering from a disease or condition by determining whether or not a gene or genes in cells of the patient (in some cases including both normal and disease cells, such as cancer cells) contain at least one sequence variance which is indicative of the effectiveness of the treatment of the disease or condition. The gene is one specified herein, in particular one listed in a Table or list herein. Preferably the at least one variance includes a plurality of variances which may provide a haplotype or haplotypes. Preferably the joint presence of the plurality of variances is indicative of the potential effectiveness of the treatment in a patient having such plurality of variances. The plurality of variances may each be indicative of the potential effectiveness of the treatment, and the effects of the individual variances may be independent or additive, or the plurality of variances may be indicative of the potential effectiveness if at least 2, 3, 4, or more appear jointly. The plurality of variances may also be combinations of these relationships. The plurality of variances may include variances from one, two, three or more gene loci.

In a related aspect, the invention concerns a method for providing a correlation between a patient genotype and effectiveness of a treatment, by determining the presence or absence of a particular known variance or variances in cells of a patient for a gene of this invention, and providing a result indicating the expected effectiveness of a treatment for a disease or condition. The result may be formulated by comparing the genotype of the patient with a list of variances indicative of the effectiveness of a treatment, e.g., administration of a drug described herein. The determination may be by methods as described herein or other methods known to those skilled in the art.

In some cases, the selection of a method of treatment, i.e., a therapeutic regimen, may incorporate selection of one or more from a plurality of medical therapies. Thus, the

selection may be the selection of a method or methods which is/are more effective or less

effective than certain other therapeutic regimens (with either having varying safety

parameters). Likewise or in combination with the preceding selection, the selection may

be the selection of a method or methods which is safer than certain other methods of

5   treatment in the patient.

The selection may involve either positive selection or negative selection or both,

meaning that the selection can involve a choice that a particular method would be an

appropriate method to use and/or a choice that a particular method would be an

inappropriate method to use. Thus, in certain embodiments, the presence of the at least

10   one variance is indicative that the treatment will be effective or otherwise beneficial (or

more likely to be beneficial) in the patient. Stating that the treatment will be effective

means that the probability of beneficial therapeutic effect is greater than in a person not

having the appropriate presence or absence of particular variances. In other

embodiments, the presence of the at least one variance is indicative that the treatment will

15   be ineffective or contra-indicated for the patient. For example, a treatment may be

contra-indicated if the treatment results, or is more likely to result, in undesirable side

effects, or an excessive level of undesirable side effects. A determination of what

constitutes excessive side-effects will vary, for example, depending on the disease or

condition being treated, the availability of alternatives, the expected or experienced

20   efficacy of the treatment, and the tolerance of the patient. As for an effective treatment,

this means that it is more likely that a desired effect will result from the treatment

administration in a patient with a particular variance or variances than in a patient who

has a different variance or variances. Also in preferred embodiments, the presence of the

at least one variance is indicative that the treatment is effective but results in undesirable

25   effects or outcomes, e.g., has undesirable side-effects.

In reference to response to a treatment, the term "tolerance" refers to the ability

of a patient to accept a treatment, based, e.g., on deleterious effects and/or effects on

lifestyle. Frequently, the term principally concerns the patients perceived magnitude of

deleterious effects such as nausea, weakness, dizziness, and diarrhea, among others. Such

30   experienced effects can, for example, be due to general or cell-specific toxicity, activity

on non-target cells, cross-reactivity on non-target cellular constituents (non-mechanism

based), and/or side-effects of activity on the target cellular subsitutuent (mechanism

based), or the cause of toxicity may not be understood. In any of these circumstances one

may identify an association between the undesirable effects and variances in specific genes.

Adverse responses to drugs constitute a major medical problem, as shown in two recent meta-analyses (Lazarou, J. et al, Incidence of adverse drug reactions in

5   hospitalized patients: a meta-analysis of prospective studies, JAMA 279:1200-1205, 1998; Bonn, Adverse drug reactions remain a major cause of death, Lancet 351:1183, 1998). An estimated 2.2 million hospitalized patients in the United Stated had serious adverse drug reactions in 1994, with an estimated 106,000 deaths (Lazarou et al.). To the extent that some of these adverse events are due to genetically encoded biochemical

10 diversity among patients in pathways that effect drug action, the identification of variances that are predictive of such effects will allow for more effective and safer drug use.

In embodiments of this invention, the variance or variant form or forms of a gene is/are associated with a specific response to a drug. The frequency of a specific variance

15 or variant form of the gene may correspond to the frequency of an efficacious response to administration of a drug. Alternatively, the frequency of a specific variance or variant form of the gene may correspond to the frequency of an adverse event resulting from administration of a drug. Alternatively the frequency of a specific variance or variant form of a gene may not correspond closely with the frequency of a beneficial or adverse

20 response, yet the variance may still be useful for identifying a patient subset with high response or toxicity incidence because the variance may account for only a fraction of the patients with high response or toxicity. Preferably, the drug will be effective in more than 20% of individuals with one or more specific variances or variant forms of the gene, more preferably in 40% and most preferably in >60%. In other embodiments, the drug

25 will be toxic or create clinically unacceptable side effects in more than 10% of individuals with one or more variances or variant forms of the gene, more preferably in >30%, more preferably in >50%, and most preferably in >70% or in more than 90%.

Also in other embodiments, the method of selecting a treatment includes eliminating a treatment, where the presence or absence of the at least one variance is

30 indicative that the treatment will be ineffective or contra-indicated. In other preferred embodiments, in cases in which undesirable side-effects may occur or are expected to occur from a particular therapeutic treatment, the selection of a method of treatment can include identifying both a first and second treatment, where the first treatment is effective

to treat the disease or condition, and the second treatment reduces a deleterious effect of the first treatment.

The phrase "eliminating a treatment" refers to removing a possible treatment from consideration, e.g., for use with a particular patient based on the presence or absence of a particular variance(s) in one or more genes in cells of that patient, or to stopping the administration of a treatment which was in the course of administration.

Usually, the treatment will involve the administration of a compound preferentially active in patients with a form or forms of a gene, where the gene is one identified herein. The administration may involve a combination of compounds. Thus, in preferred embodiments, the method involves identifying such an active compound or combination of compounds, where the compound is less active or is less safe or both when administered to a patient having a different form of the gene. In preferred embodiments, the compound is a compound in a drug class identified in the 1999 Physicians' Desk Reference (53rd edition), Medical Economics Data, 1998, the PharmaProjects database, the IMS database or identified herein, e.g., in an exemplary drug table herein (see, e.g., Examples 6, 8, and 9 and Tables 7 and 9 herein).

Also in preferred embodiments, the method of selecting a treatment involves selecting a method of administration of a compound, combination of compounds, or pharmaceutical composition, for example, selecting a suitable dosage level and/or frequency of administration, and/or mode of administration of a compound. The method of administration can be selected to provide better, preferably maximum therapeutic benefit. In this context, "maximum" refers to an approximate local maximum based on the parameters being considered, not an absolute maximum.

Also in this context, a "suitable dosage level" refers to a dosage level which provides a therapeutically reasonable balance between pharmacological effectiveness and deleterious effects. Often this dosage level is related to the peak or aveage serum levels resulting from administration of a drug at the particular dosage level.

Similarly, a "frequency of administration" refers to how often in a specified time period a treatment is administered, e.g., once, twice, or three times per day, every other day, once per week, etc. For a drug or drugs, the frequency of administration is generally selected to achieve a pharmacologically effective average or peak serum level without excessive deleterious effects (and preferably while still being able to have reasonable patient compliance for self-administered drugs). Thus, it is desirable to maintain the

serum level of the drug within a therapeutic window of concentrations for the greatest percentage of time possible without such deleterious effects as would cause a prudent physician to reduce the frequency of administration for a particular dosage level.

A particular gene or genes can be relevant to more than one disease or condition,
5   for example, the gene or genes can have a role in the initiation, development, course, treatment, treatment outcomes, or health-related quality of life outcomes of a number of different diseases, disorders, or conditions. Thus, in preferred embodiments, the disease or condition or treatment of the disease or condition is any which involves a particular gene. Preferably the gene is a gene identified herein.

10   Determining the presence of a particular variance or plurality of variances in a particular gene in a patient can be performed in a variety of ways. In preferred embodiments, the detection of the presence or absence of at least one variance involves amplifying a segment of nucleic acid including at least one of the at least one variances. Preferably a segment of nucleic acid to be amplified is 500 nucleotides or less in length,
15   more preferably 100 nucleotides or less, and most preferably 45 nucleotides or less. Also, preferably the amplified segment or segments includes a plurality of variances, or a plurality of segments of a gene or of a plurality of genes.

In another aspect determining the presence of a set of variances in a specific gene may entail a haplotyping test that requires allele-specific amplification of a large DNA
20   segment of no greater than 20,000 nucleotides, preferably no greater than 10,000 nucleotides and more preferably no greater than 5,000 nucleotides. Alternatively one allele may be enriched by methods other than amplification prior to determining genotypes at specific variant positions on the enriched allele as a way of determining haplotypes. Preferably the determination of the presence or absence of a variance
25   involves determining the sequence of the variance site or sites by methods such as chain terminating DNA sequencing or minisequencing, or by oligonucleotide hybridization or by mass spectrometry.

The term "genotype" in the context of this invention refers to the particular allelic form of a gene, which can be defined by the particular nucleotide(s) present in a
30   nucleic acid sequence at a particular site(s).

In preferred embodiments, the detection of the presence or absence of the at least one variance involves contacting a nucleic acid sequence corresponding to one of the genes identified above or a product of such a gene with a probe. The probe is able to

distinguish a particular form of the gene or gene product or the presence or a particular
variance or variances, e.g., by differential binding or hybridization. Thus, exemplary
probes include nucleic acid hybridization probes, peptide nucleic acid probes, nucleotide-
containing probes which also contain at least one nucleotide analog, and antibodies, e.g.,

5   monoclonal antibodies, and other probes as discussed herein. Those skilled in the art are
familiar with the preparation of probes with particular specificities. Those skilled in the
art will recognize that a variety of variables can be adjusted to optimize the
discrimination between two variant forms of a gene, including changes in salt
concentration, temperature, pH and addition of various compounds that affect the

10   differential affinity of GC vs. AT base pairs, such as tetramethyl ammonium chloride.
(See Current Protocols in Molecular Biology by F. M. Ausubel, R. Brent, R. E. Kingston,
D. D. Moore, J.G. Seidman, K. Struhl and V. B. Chanda (Editors), John Wiley & Sons.)

     In other preferred embodiments, determining the presence or absence of the at
least one variance involves sequencing at least one nucleic acid sequence. The

15   sequencing involves sequencing of a portion or portions of a gene and/or portions of a
plurality of genes which includes at least one variance site, and may include a plurality of
such sites. Preferably, the portion is 500 nucleotides or less in length, more preferably
100 nucleotides or less, and most preferably 45 nucleotides or less in length. Such
sequencing can be carried out by various methods recognized by those skilled in the art,

20   including use of dideoxy termination methods (e.g., using dye-labeled dideoxy
nucleotides) and the use of mass spectrometric methods. In addition, mass spectrometric
methods may be used to determine the nucleotide present at a variance site. In preferred
embodiments in which a plurality of variances is determined, the plurality of variances
can constitute a haplotype or haplotypes.

25   The terms "variant form of a gene", "form of a gene", or "allele" refer to one
specific form of a gene in a population, the specific form differing from other forms of
the same gene in the sequence of at least one, and frequently more than one, variant sites
within the sequence of the gene. The sequences at these variant sites that differ between
different alleles of the gene are termed "gene sequence variances" or "variances" or

30   "variants". The term "alternative form" refers to an allele that can be distinguished from
other alleles by having distinct variances at at least one, and frequently more than one,
variant sites within the gene sequence. Other terms known in the art to be equivalent
include mutation and polymorphism, although mutation is often used to refer to an allele

associated with a deleterious phenotype. In preferred aspects of this invention, the variances are selected from the group consisting of the variances listed in the variance tables herein or in a patent or patent application referenced and incorporated by reference in this disclosure. In the methods utilizing variance presence or absence, reference to the

5   presence of a variance or variances means particular variances, i.e., particular nucleotides at particular polymorphic sites, rather than just the presence of any variance in the gene.

Variances occur in the human genome at approximately one in every 500 – 1,000 bases within the human genome when two alleles are compared. When multiple alleles from unrelated individuals are compared the frequency of variant sites increases. At most

10 variant sites there are only two alternative nucleotides involving the substitution of one base for another or the insertion/deletion of one or more nucleotides. Within a gene there may be several variant sites. Variant forms of the gene or alternative alleles can be distinguished by the presence of alternative variances at a single variant site, or a combination of several different variances at different sites (haplotypes).

15 It is estimated that there are 3,300,000,000 bases in the sequence of a single haploid human genome. All human cells except germ cells are normally diploid. Each gene in the genome may span 100-10,000,000 bases of DNA sequence or 100-20,000 bases of mRNA. It is estimated that there are between 60,000 and 120,000 genes in the human genome. The "identification" of genetic variances or variant forms of a gene

20 involves the discovery of variances that are present in a population. The identification of variances is required for development of a diagnostic test to determine whether a patient has a variant form of a gene that is known to be associated with a disease, condition, or predisposition or with the efficacy or safety of the drug. Identification of previously undiscovered genetic variances is distinct from the process of "determining" the status of

25 known variances by a diagnostic test. The present invention provides exemplary variances in genes listed in the gene tables, as well as methods for discovering additional variances in those genes and a comprehensive written description of such additional possible variances. Also described are methods for DNA diagnostic tests to determine the DNA sequence at a particular variant site or sites.

30 The process of "identifying" or discovering new variances involves comparing the sequence of at least two alleles of a gene, more preferably at least 10 alleles and most preferably at least 50 alleles, (keeping in mind that each somatic cell has two alleles). The analysis of large numbers of individuals to discover variances in the gene sequence

between individuals in a population will result in detection of a greater fraction of all the variances in the population. Preferably the process of identifying reveals whether there is a variance within the gene; more preferably identifying reveals the location of the variance within the gene; more preferably identifying provides knowledge of the

5   sequence of the nucleic acid sequence of the variance, and most preferably identifying provides knowledge of the combination of different variances that comprise specific variant forms of the gene or alleles. In identifying new variances it is often useful to screen different population groups based on racial, ethnic, gender, and/or geographic origin because particular variances may differ in frequency between such groups. It may

10   also be useful to screen DNA from individuals with a particular disease or condition of interest because they may have a higher frequency of certain variances than the general population.

            The process of determining involves using diagnostic tests for specific variances or variant forms of the gene (or genes) that have been identified within the gene. It will

15   be apparent that such diagnostic tests can only be performed after variances and variant forms of the gene have been identified. Identification of variances can be performed by a variety of methods, alone or in combination, including, for example, DNA sequencing, SSCP, heteroduplex analysis, denaturing gradient gel electrophoresis (DGGE), heteroduplex cleavage (either enzymatic as with T4 Endonuclease 7, or chemical as with

20   osmium tetroxide and hydroxylamine), computational methods (described herein), and other methods described herein as well as others known to those skilled in the art. (See, for example: Cotton, R.G.H., Slowly but surely towards better scanning for mutations, Trends in Genetics 13(2):43-6, 1997, or Current Protocols in Human Genetics by N. C. Dracopoli, J. L. Haines, B. R. Korf, D. T. Moir , C. C. Morton, C. E. Seidman, J.G.

25   Seidman, D. R. Smith and A. Boyle (Editors), John Wiley & Sons.)

            In the context of this invention, the term "analyzing a sequence" refers to determining at least some sequence information about the sequence, e.g., determining the nucleotides present at particular sites in the sequence or determining the base sequence of all of a portion of the particular sequence.

30   In the context of this invention, the term "haplotype" refers to a *cis* arrangement of two or more polymorphic nucleotides, i.e., variances, on a particular chromosome, e.g., in a particular gene. The haplotype preserves the information of the phase of the

polymorphic nucleotides – that is, which set of variances were inherited from one parent, and which from the other.

In preferred embodiments of this invention, the frequency of the variance or variant form of the gene in a population is known. Measures of frequency known in the art include "allele frequency", namely the fraction of genes in a population that have one specific variance or set of variances. The allele frequencies for any gene should sum to 1. Another measure of frequency known in the art is the "heterozygote frequency" namely, the fraction of individuals in a population who carry two alleles, or two forms of a particular variance or variant form of a gene, one inherited from each parent.

Alternatively, the number of individuals who are homozygous for a particular form of a gene may be a useful measure. The relationship between allele frequency, heterozygote frequency, and homozygote frequency is described for many genes by the Hardy-Weinberg equation, which provides the relationship between allele frequency, heterozygote frequency and homozygote frequency in a freely breeding population at equilibrium. Most human variances are substantially in Hardy-Weinberg equilibrium. In a preferred aspect of this invention, the allele frequency, heterozygote frequency, or homozygote frequency are determined experimentally. Preferably a variance has an allele frequency of at least 0.01, more preferably at least 0.05, still more preferably at least 0.10. However, the allele may have a frequency as low as 0.001 if the associated phenotype is a rare form of toxic reaction to the treatment or drug.

In this regard, "population" refers to a geographically, ethnically, racially, gender, and/or culturally defined group of individuals or a group of individuals with a particular disease or condition or individuals that may be treated with a specific drug. In most cases a population will preferably encompass at least ten thousand, one hundred thousand, one million, ten million, or more individuals, with the larger numbers being more preferable. In a preferred aspect of this invention, the population refers to individuals with a specific disease or condition that may be treated with a specific drug. In an aspect of this invention, the allele frequency, heterozygote frequency, or homozygote frequency of a specific variance or variant form of a gene is known. In preferred embodiments of this invention, the frequency of one or more variances that may predict response to a treatment is determined in one or more populations using a diagnostic test.

It should be emphasized that it is currently not generally practical to study entire gene sequences in entire populations to establish the association between a specific

disease or condition and a specific variance or variant form of the gene. Such studies are commonly performed in controlled clinical trials using a limited number of patients that are considered to be representative of the population with the disease.

In the context of this invention, the term "probe" refers to a molecule which can detectably distinguish between target molecules differing in structure. Detection can be accomplished in a variety of different ways depending on the type of probe used and the type of target molecule. Thus, for example, detection may be based on discrimination of activity levels of the target molecule, but preferably is based on detection of specific binding. Examples of such specific binding include antibody binding and nucleic acid probe hybridization. Thus, for example, probes can include enzyme substrates, antibodies and antibody fragments, and nucleic acid hybridization probes. Thus, in preferred embodiments, the detection of the presence or absence of the at least one variance involves contacting a nucleic acid sequence which includes a variance site with a probe, preferably a nucleic acid probe, where the probe preferentially hybridizes with a form of the nucleic acid sequence containing a complementary base at the variance site as compared to hybridization to a form of the nucleic acid sequence having a non-complementary base at the variance site, where the hybridization is carried out under selective hybridization conditions. Such a nucleic acid hybridization probe may span two or more variance sites. Unless otherwise specified, a nucleic acid probe can include one or more nucleic acid analogs, labels or other substituents or moieties so long as the base-pairing function is retained.

As is generally understood, administration of a particular treatment, e.g., administration of a therapeutic compound or combination of compounds, is chosen depending on the disease or condition which is to be treated. Thus, in certain preferred embodiments, the disease or condition is one for which administration of a treatment is expected to provide a therapeutic benefit; in certain embodiments, the compound is a compound identified herein, e.g., in a drug table such as Tables 7 and 9.

As used herein, the terms "effective" and "effectiveness" includes both pharmacological effectiveness and physiological safety. Pharmacological effectiveness refers to the ability of the treatment to result in a desired biological effect in the patient. Physiological safety refers to the level of toxicity, or other adverse physiological effects at the cellular, organ and/or organism level (often referred to as side-effects) resulting from administration of the treatment. On the other hand, the term "ineffective" indicates

that a treatment does not provide sufficient pharmacological effect to be therapeutically useful, even in the absence of deleterious effects, at least in the total (unstratified) population. (Such a treatment may be effective in a subgroup that can be identified by the presence of one or more sequence variances or alleles.) "Less effective" means that

5   the treatment results in a therapeutically significant lower level of pharmacological effectiveness and/or a therapeutically greater level of adverse physiological effects.

Thus, in connection with the administration of a drug, a drug which is "effective against" a disease or condition indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of

10   patients, such as a improvement of symptoms, a cure, a reduction in disease load, reduction in tumor mass or cell numbers, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating the particular type of disease or condition.

The term "deleterious effects" refers to physical effects in a patient caused by

15   administration of a treatment which are regarded as medically undesirable. Thus, for example, deleterious effects can include a wide spectrum of toxic effects injurious to health such as death of normal cells when only death of diseased cells is desired, nausea, fever, inability to retain food, dehydration, damage to critical organs such as renal tubular necrosis, fatty liver or pulmonary fibrosis, among many others. In this regard, the term

20   "contra-indicated" means that a treatment results in deleterious effects such that a prudent medical doctor treating such a patient would regard the treatment as unsuitable for administration. Major factors in such a determination can include, for example, availability and relative advantages of alternative treatments, consequences of non-treatment, and permanency of deleterious effects of the treatment.

25   It is recognized that many treatment methods, e.g., administration of certain compounds or combinations of compounds, produces side-effects or other deleterious effects in patients. Such effects can limit or even preclude use of the treatment method in particular patients, or may even result in irreversible injury, dysfunction, or death of the patient. Thus, in certain embodiments, the variance information is used to select both a

30   first method of treatment and a second method of treatment. Usually the first treatment is a primary treatment which provides a physiological effect directed against the disease or condition or its symptoms. The second method is directed to reducing or eliminating one or more deleterious effects of the first treatment, e.g., to reduce a general toxicity or to

reduce a side effect of the primary treatment. Thus, for example, the second method can be used to allow use of a greater dose or duration of the first treatment, or to allow use of the first treatment in patients for whom the first treatment would not be tolerated or would be contra-indicated in the absence of a second method to reduce deleterious

5   effects.

In a related aspect, the invention provides a method for selecting a method of treatment for a patient suffering from a disease or condition by comparing at least one variance in at least one gene in the patient, with a list of variances in the gene or genes

10   which are indicative of the effectiveness of at least one method of treatment. Preferably the comparison involves a plurality of variances or a haplotype indicative of the effectiveness of at least one method of treatment. Also, preferably the list of variances includes a plurality of variances.

Similar to the above aspect, in preferred embodiments the at least one method of

15   treatment involves the administration of a compound effective in at least some patients with a disease or condition; the presence or absence of the at least one variance is indicative that the treatment will be effective in the patient; and/or the presence or absence of the at least one variance is indicative that the treatment will be ineffective or contra-indicated in the patient; and/or the treatment is a first treatment and the presence or

20   absence of the at least one variance is indicative that a second treatment will be beneficial to reduce a deleterious effect of the first treatment; and/or the at least one treatment is a plurality of methods of treatment. For a plurality of treatments, preferably the selecting involves determining whether any of the methods of treatment will be more effective than at least one other of the plurality of methods of treatment. Yet other embodiments are

25   provided as described for the preceding aspect in connection with methods of treatment using administration of a compound; treatment of various diseases, and variances in particular genes.

In the context of variance information in the methods of this invention, the term "list" refers to one or more variances which have been identified for a series or genes of

30   potential importance in accounting for inter-individual variation in treatment response. Preferably there is a plurality of variances for the gene or genes, preferably a plurality of variances for a particular gene. Preferably the list is recorded in written or electronic form. For example, variances are recorded in Tables 3, 4, and 10 and additional gene

variance identification tables herein in a form which allows comparison with other variance information.

In addition to the basic method of treatment, often the mode of administration of a
5   given compound as a treatment for a disease or condition in a patient is significant in determining the course and/or outcome of the treatment for the patient. Thus, the invention also provides a method for selecting a method of administration of a compound to a patient suffering from a disease or condition, by determining the presence or absence of at least one variance in cells of the patient in a gene which is a gene selected from the
10   genes identified in a gene table or list below, where such presence or absence is indicative of an appropriate method of administration of the compound. Preferably, the selection of a method of treatment (a treatment regimen) involves selecting a dosage level or frequency of administration or route of administration of the compound or combinations of those parameters. In preferred embodiments, two or more compounds are to be
15   administered, and the selecting involves selecting a method of administration for one, two, or more than two of the compounds, jointly, concurrently, or separately. As understood by those skilled in the art, such plurality of compounds is often used in combination therapy, and thus may be formulated in a single drug, or may be separate drugs administered concurrently, serially, or separately. Other embodiments are as
20   indicated above for selection of second treatment methods, methods of identifying variances, and methods of treatment as described for aspects above.

In another aspect, the invention provides a method for selecting a patient for administration of a method of treatment for a disease or condition, or of selecting a
25   patient for a method of administration of a treatment, by comparing the presence or absence of at least one variance in a gene as identified above in cells of a patient, with a list of variances in the gene, where the presence or absence of the at least one variance is indicative that the treatment or method of administration will be effective in the patient. If the at least one variance is present in the patient's cells, then the patient is selected for
30   administration of the treatment.
In preferred embodiments, the disease or the method of treatment is as described in aspects above, specifically including, for example, those described for selecting a method of treatment

In another aspect, the invention provides a method for identifying a subset of patients with enhanced or diminished response or tolerance to a treatment method or a method of administration of a treatment where the treatment is for a disease or condition in the patient. The method involves correlating one or more variances in one or more

5 genes in a plurality of patients with response to a treatment or a method of administration of a treatment. The correlation may be performed by determining the one or more variances in the one or more genes in the plurality of patients and correlating the presence or absence of each of the variances (alone or in various combinations) with the patient's response to treatment. The variances may be previously known to exist or may also be

10 determined in the present method or combinations of prior information and newly determined information may be used. The enhanced or diminished response should be statistically significant, preferably such that $p = 0.10$ or less, more preferably 0.05 or less, and most preferably 0.02 or less. A positive correlation between the presence of one or more variances and an enhanced response to treatment is indicative that the treatment is

15 particularly effective in the group of patients having those variances. A positive correlation of the presence of the one or more variances with a diminished response to the treatment is indicative that the treatment will be less effective in the group of patients having those variances. Such information is useful, for example, for selecting or de-selecting patients for a particular treatment or method of administration of a treatment, or

20 for demonstrating that a group of patients exists for which the treatment or method of treatment would be particularly beneficial or contra-indicated. Such demonstration can be beneficial, for example, for obtaining government regulatory approval for a new drug or a new use of a drug.

In preferred embodiments, the variances are in particular genes, or are particular

25 variances described herein. Also, preferred embodiments include drugs, treatments, variance identification or determination, determination of effectiveness, lists, and/or diseases as described for aspects above or otherwise described herein.

In preferred embodiments, the correlation of patient responses to therapy according to patient genotype is carried out in a clinical trial, e.g., as described herein

30 according to any of the variations described.. Detailed description of methods for associating variances with clinical outcomes using clinical trials are provided below.

As indicated above, in aspects of this invention involving selection of a patient for a treatment, selection of a method or mode of administration of a treatment, and selection of a patient for a treatment or a method of treatment, the selection may be positive selection or negative selection. Thus, the methods can include eliminating a treatment for

5   a patient, eliminating a method or mode of administration of a treatment to a patient, or elimination of a patient for a treatment or method of treatment.

Also, in methods involving identification and/or comparison of variances present in a gene of a patient, the methods can involve such identification or comparison for a plurality of genes. Preferably, the genes are functionally related to the same disease or

10   condition, or to the aspect of disease pathophysiology that is being subjected to pharmacological manipulation by the treatment (e.g. a drug), or to the activation or inactivation of the drug, and more preferably the genes are involved in the same biochemical process or pathway.

15   In another aspect, the invention provides a method for identifying the forms of a gene in an individual, where the gene is one specified as for aspects above, by determining the presence or absence of at least one variance in the gene. In preferred embodiments, the at least one variance includes at least one variance selected from the group of variances identified in variance tables herein. Preferably, the presence or

20   absence of the at least one variance is indicative of the effectiveness of a therapeutic treatment in a patient suffering from a disease or condition and having cells containing the at least one variance.

The presence or absence of the variances can be determined in any of a variety of ways as recognized by those skilled in the art. For example, the nucleotide sequence of at

25   least one nucleic acid sequence which includes at least one variance site (or a complementary sequence) can be determined, such as by chain termination methods, hybridization methods or by mass spectrometric methods. Likewise, in preferred embodiments, the determining involves contacting a nucleic acid sequence or a gene product of one of one of the genes with a probe which specifically identifies the presence

30   or absence of a form of the gene. For example, a probe, e.g., a nucleic acid probe, can be used which specifically binds, e.g., hybridizes, to a nucleic acid sequence corresponding to a portion of the gene and which includes at least one variance site under selective

binding conditions. As described for other aspects, determining the presence or absence of at least two variances can constitute determining a haplotype or haplotypes.

Other preferred embodiments involve variances related to types of treatment, drug responses, diseases, nucleic acid sequences, and other items related to variances and
5 variance determination as described for aspects above.

In yet another aspect, the invention provides a pharmaceutical composition which includes a compound which has a differential effect in patients having at least one copy, or alternatively, two copies of a form of a gene as identified for aspects above and a
10 pharmaceutically acceptable carrier, excipient, or diluent. The composition is adapted to be preferentially effective to treat a patient with cells containing the one, two, or more copies of the form of the gene.

In preferred embodiments of aspects involving pharmaceutical compositions, active compounds, or drugs, the material is subject to a regulatory limitation or restriction
15 on approved uses or indications, e.g., by the U.S. Food and Drug Administration (FDA), limiting approved use of the composition to patients having at least one copy of the particular form of the gene which contains at least one variance. Alternatively, the composition is subject to a regulatory limitation or restriction on approved uses indicating that the composition is not approved for use or should not be used in patients having at
20 least one copy of a form of the gene including at least one variance. Also in preferred embodiments, the composition is packaged, and the packaging includes a label or insert indicating or suggesting beneficial therapeutic approved use of the composition in patients having one or two copies of a form of the gene including at least one variance. Alternatively, the label or insert limits approved use of the composition to patients having
25 zero or one or two copies of a form of the gene including at least one variance. The latter embodiment would be likely where the presence of the at least one variance in one or two copies in cells of a patient means that the composition would be ineffective or deleterious to the patient. Also in preferred embodiments, the composition is indicated for use in treatment of a disease or condition which is one of those identified for aspects above.
30 Also in preferred embodiments, the at least one variance includes at least one variance from those identified herein.

The term "packaged" means that the drug, compound, or composition is prepared in a manner suitable for distribution or shipping with a box, vial, pouch, bubble pack, or

other protective container, which may also be used in combination. The packaging may have printing on it and/or printed material may be included in the packaging.

In preferred embodiments, the drug is selected from the drug classes or specific exemplary drugs identified in an example, in a table or list herein, and is subject to a

5 regulatory limitation or suggestion or warning as described above that limits or suggests limiting approved use to patients having specific variances or variant forms of a gene identified in Examples or in a gene list provided below in order to achieve maximal benefit and avoid toxicity or other deleterious effect.

A pharmaceutical composition can be adapted to be preferentially effective in a

10 variety of ways. In some cases, an active compound is selected which was not previously known to be differentially active, or which was not previously recognized as a potential therapeutic compound. In some cases, the concentration of an active compound which has differential activity can be adjusted such that the composition is appropriate for administration to a patient with the specified variances. For example, the presence of a

15 specified variance may allow or require the administration of a much larger dose, which would not be practical with a previously utilized composition. Conversely, a patient may require a much lower dose, such that administration of such a dose with a prior composition would be impractical or inaccurate. Thus, the composition may be prepared in a higher or lower unit dose form, or prepared in a higher or lower concentration of the

20 active compound or compounds. In yet other cases, the composition can include additional compounds needed to enable administration of a particular active compound in a patient with the specified variances, which was not in previous compositions, e.g., because the majority of patients did not require or benefit from the added component.

The term "differential" or "differentially" generally refers to a statistically

25 significant different level in the specified property or effect. Perferably, the difference is also functionally significant. Thus, "differential binding or hybridization" is sufficient difference in binding or hybridization to allow discrimination using an appropriate detection technique. Likewise, "differential effect" or "differentially active" in connection with a therapeutic treatment or drug refers to a difference in the level of the

30 effect or activity which is distinguishable using relevant parameters and techniques for the effect or activity being considered. Preferably the difference in effect or activity is also sufficient to be clinically significant, such that a corresponding difference in the

course of treatment or treatment outcome would be expected, at least on a probabilistic basis.

Also usefully provided in the present invention are probes which specifically
5   recognize a nucleic acid sequence corresponding to a variance or variances in a gene or a product expressed from the gene, and are able to distinguish a variant form of the sequence or gene or gene product from one or more other variant forms of that sequence, gene, or gene product under selective conditions. Those skilled in the art recognize and understand the identification or determination of selective conditions for particular probes
10   or types of probes. An exemplary type of probe is a nucleic acid hybridization probe, which will selectively bind under selective binding conditions to a nucleic acid sequence or a gene product corresponding to one or the genes identified for aspects above. Another type of probe is a peptide or protein, e.g., an antibody or antibody fragment which specifically or preferentially binds to a polypeptide expressed from a particular form of a
15   gene as characterized by the presence or absence of at least one variance. Thus, in another aspect, the invention concerns such probes. In the context of this invention, a "probe" is a molecule, commonly a nucleic acid, though also potentially a protein, carbohydrate, polymer, or small molecule, that is capable of binding to one variance or variant form of the gene or gene product to a greater extent than to a form of the gene
20   having a different base at one or more variance sites, such that the presence of the variance or variant form of the gene can be determined. Preferably the probe distinguishes at least one variance identified in Examples, tables or lists below. Preferably the probe also has specificity for the particular gene or gene product, at least to an extent such that binding to other genes or gene products does not prevent use of the
25   assay to identify the presence or absence of the particular variance or variances of interest.

In preferred embodiments, the probe is an antibody or antibody fragment. Such antibodies may be polyclonal or monoclonal antibodies, and can be prepared by methods well-known in the art. In preferred embodiments, the probe is a nucleic acid probe at
30   least 15, preferably at least 17 nucleotides in length, more preferably at least 20 or 22 or 25, preferably 500 or fewer nucleotides in length, more preferably 200 or 100 or fewer, still more preferably 50 or fewer, and most preferably 30 or fewer. In preferred embodiments, the probe has a length in a range between from any one of the above

lengths to any other of the above lengths (including endpoints). The probe specifically hybridizes under selective hybridization conditions to a nucleic acid sequence corresponding to a portion of one of the genes identified in connection with above aspects. The nucleic acid sequence includes at least one and preferably two or more
5   variance sites. Also in preferred embodiments, the probe has a detectable label, preferably a fluorescent label. A variety of other detectable labels are known to those skilled in the art. Such a nucleic acid probe can also include one or more nucleic acid analogs.

In preferred embodiments, the probe is an antibody or antibody fragment which
10   specifically binds to a gene product expressed from a form of one of the above genes, where the form of the gene has at least one specific variance with a particular base at the variance site, and preferably a plurality of such variances.

In connection with nucleic acid probe hybridization, the term "specifically hybridizes" indicates that the probe hybridizes to a sufficiently greater degree to the
15   target sequence than to a sequence having a mismatched base at at least one variance site to allow distinguishing such hybridization. The term "specifically hybridizes" thus means that the probe hybridizes to the target sequence, and not to non-target sequences, at a level which allows ready identification of probe/target sequence hybridization under selective hybridization conditions. Thus, "selective hybridization conditions" refer to
20   conditions which allow such differential binding. Similarly, the terms "specifically binds" and "selective binding conditions" refer to such differential binding of any type of probe, e.g., antibody probes, and to the conditions which allow such differential binding. Typically hybridization reactions to determine the status of variant sites in patient samples are carried out with two different probes, one specific for each of the
25   (usually two) possible variant nucleotides. The complementary information derived from the two separate hybridization reactions is useful in corroborating the results.

Likewise, the invention provides an isolated, purified or enriched nucleic acid sequence of 15 to 500 nucleotides in length, preferably 15 to 100 nucleotides in length,
30   more preferably 15 to 50 nucleotides in length, and most preferably 15 to 30 nucleotides in length, which has a sequence which corresponds to a portion of one of the genes identified for aspects above. Preferably the lower limit for the preceding ranges is 17, 20, 22, or 25 nucleotides in length. In other embodiments, the nucleic acid sequence is 30 to

300 nucleotides in length, or 45 to 200 nucleotides in length, or 45 to 100 nucleotides in length. The nucleic acid sequence includes at least one variance site. Such sequences can, for example, be amplification products of a sequence which spans or includes a variance site in a gene identified herein. Likewise, such a sequence can be a primer which is able

5   to bind to or extend through a variance site in such a gene. Yet another example is a nucleic acid hybridization probe comprised of such a sequence. In such probes, primers, and amplification products, the nucleotide sequence can contain a sequence or site corresponding to a variance site or sites, for example, a variance site identified herein. Preferably the presence or absence of a particular variant form in the heterozygous or

10   homozygous state is indicative of the effectiveness of a method of treatment in a patient.

Typically primers are utilized in pairs. Primers can be designed or selected by methods well-known to those skilled in the art based on nucleotide sequences corresponding to at least a portion or a gene identified herein. The primer or primers hybridizes to or allows amplification (e.g., using the polymerase chain reaction) through a

15   nucleic acid sequence containing at least one sequence variance. Preferably such primers hybridize to a sequence not more than 300 nucleotides, more preferably not more than 200 nucleotides, still more preferably not more than 100 nucleotides, and most preferably not more than 50 nucleotides away from a variance site which is to be analyzed. Preferably, a primer is 100 nucleotides or fewer in length, more preferably 50 nucleotides

20   or fewer, still more preferable 30 nucleotides or fewer, and most preferably 20 or fewer nucleotides in length.

In reference to nucleic acid sequences which "correspond" to a gene, the term "correspond" refers to a nucleotide sequence relationship, such that the nucleotide sequence has a nucleotide sequence which is the same as the reference gene or an

25   indicated portion thereof, or has a nucleotide sequence which is exactly complementary in normal Watson-Crick base pairing, or is an RNA equivalent of such a sequence, e.g., a mRNA, or is a cDNA derived from an mRNA of the gene.

In a related aspect, the invention provides a kit containing at least one probe or at

30   least one primer or both (e.g., as described above) corresponding to a gene or genes of this invention. The kit is preferably adapted and configured to be suitable for identification of the presence or absence of a particular variance or variances, which can include or consist of sequence a nucleic acid sequence corresponding to a portion of a

gene. The kit may also contain a plurality of either or both of such probes and/or primers, e.g., 2, 3, 4, 5, 6, or more of such probes and/or primers. Preferably the plurality of probes and/or primers are adapted to provide detection of a plurality of different sequence variances in a gene or plurality of genes, e.g., in 2, 3, 4, 5, or more genes or to sequence a

5 nucleic acid sequence including at least one variance site in a gene or genes. Preferably one or more of the variance or variances to be detected are correlated with variability in a treatment response or tolerance, and are preferably indicative of an effective response to a treatment. In preferred embodiments, the kit contains components (e.g., probes and/or primers) adapted or useful for detection of a plurality of variances (which may be in one

10 or more genes) indicative of the effectiveness of at least one treatment, preferably of a plurality of different treatments for a particular disease or condition. It may also be desirable to provide a kit containing components adapted or useful to allow detection of a plurality of variances indicative of the effectiveness of a treatment or treatment against a plurality of diseases. The kit may also optionally contain other components, preferably

15 other components adapted for identifying the presence of a particular variance or variances. Such additional components can, for example, independently include a buffer or buffers, e.g., amplification buffers and hybridization buffers, which may be in liquid or dry form, a DNA polymerase, e.g., a polymerase suitable for carrying out PCR, and deoxy nucleotide triphosphases (dNTPs). Preferably a probe includes a detectable label,

20 e.g., a fluorescent label, enzyme label, light scattering label, or other label. Preferably the kit includes a nucleic acid or polypeptide array. The array may, for example, include a plurality of different antibodies, a plurality of different nucleic acid sequences. Sites in the array can allow capture and/or detection of nucleic acid sequences or gene products corresponding to different variances in one or more different genes. Preferably the array

25 is arranged to provide variance detection for a plurality of variances in one or more genes which correlate with the effectiveness of one or more treatments of one or more diseases.

The kit may also optionally contain instructions for use, which can include a listing of the variances correlating with a particular treatment or treatments for a disease of diseases.

30 Preferably the kit components are selected to allow detection of a variance described herein, and/or detection of a variance indicative of a treatment,e.g., administration of a drug, pointed out herein.

Additional configurations for kits of this invention will be apparent to those skilled in the art.

In another aspect, the invention provides a method for determining a genotype of

5   an individual in relation to one or more variances in one or more of the genes identified in above aspects by using mass spectrometric determination of a nucleic acid sequence which is a portion of a gene identified for other aspects of this invention or a complementary sequence. Such mass spectrometric methods are known to those skilled in the art. In preferred embodiments, the method involves determining the presence or

10 absence of a variance in a gene; determining the nucleotide sequence of the nucleic acid sequence; the nucleotide sequence is 100 nucleotides or less in length, preferably 50 or less, more preferably 30 or less, and still more preferably 20 nucleotides or less. In general, such a nucleotide sequence includes at least one variance site, preferably a variance site which is informative with respect to the expected response of a patient to a

15 treatment as described for above aspects.

As indicated above, many therapeutic compounds or combinations of compounds or pharmaceutical compositions show variable efficacy and/or safety in various patients in whom the compound or compounds is administered. Thus, it is beneficial to identify

20 variances in relevant genes, e.g., genes related to the action or toxicity of the compound or compounds. Thus, in a further aspect, the invention provides a method for determining whether a compound has a differential effect due to the presence or absence of at least one variance in a gene or a variant form of a gene, where the gene is a gene identified for aspects above.

25 The method involves identifying a first patient or set of patients suffering from a disease or condition whose response to a treatment differs from the response (to the same treatment) of a second patient or set of patients suffering from the same disease or condition, and then determining whether the frequency of at least one variance in at least one gene differs in frequency between the first patient or set of patients and the second

30 patient or set of patients. A correlation between the presence or absence of the variance or variances and the response of the patient or patients to the treatment indicates that the variance provides information about variable patient response. In general, the method will involve identifying at least one variance in at least one gene. An alternative

approach is to identify a first patient or set of patients suffering from a disease or condition and having a particular genotype, haplotype or combination of genotypes or haplotypes, and a second patient or set of patients suffering from the same disease or condition that have a genotype or haplotype or sets of genotypes or haplotypes that differ

5   in a specific way from those of the first set of patients. Subsequently the extent and magnitude of clinical response can be compared between the first patient or set of patients and the second patient or set of patients. A correlation between the presence or absence of a variance or variances or haplotypes and the response of the patient or patients to the treatment indicates that the variance provides information about variable patient response

10   and is useful for the present invention.

The method can utilize a variety of different informative comparisons to identify correlations. For example a plurality of pairwise comparisons of treatment response and the presence or absence of at least one variance can be performed for a plurality of patients. Likewise, the method can involve comparing the response of at least one patient

15   homozygous for at least one variance with at least one patient homozygous for the alternative form of that variance or variances. The method can also involve comparing the response of at least one patient heterozygous for at least one variance with the response of at least one patient homozygous for the at least one variance. Preferably the heterozygous patient response is compared to both alternative homozygous forms, or the

20   response of heterozygous patients is grouped with the response of one class of homozygous patients and said group is compared to the response of the alternative homozygous group.

Such methods can utilize either retrospective or prospective information concerning treatment response variability. Thus, in a preferred embodiment, it is

25   previously known that patient response to the method of treatment is variable.

Also in preferred embodiments, the disease or condition is as for other aspects of this invention; for example, the treatment involves administration of a compound or pharmaceutical composition.

In preferred embodiments, the method involves a clinical trial, e.g., as described

30   herein. Such a trial can be arranged, for example, in any of the ways described herein, e.g., in the Detailed Description.

The present invention also provides methods of treatment of a disease or condition. Such methods combine identification of the presence or absence of particular variances with the administration of a compound; identification of the presence of particular variances with selection of a method of treatment and administration of the

5   treatment; and identification of the presence or absence of particular variances with elimination of a method of treatment based on the variance information indicating that the treatment is likely to be ineffective or contra-indicated, and thus selecting and administering an alternative treatment effective against the disease or condition. Thus, preferred embodiments of these methods incorporate preferred embodiments of such

10   methods as described for such sub-aspects.

As used herein, a "gene" is a sequence of DNA present in a cell that directs the expression of a "biologically active" molecule or "gene product", most commonly by transcription to produce RNA and translation to produce protein. The "gene product' is most commonly a RNA molecule or protein or a RNA or protein that is subsequently

15   modified by reacting with, or combining with, other constituents of the cell. Such modifications may include, without limitation, modification of proteins to form glycoproteins, lipoproteins, and phosphoproteins, or other modifications known in the art. RNA may be modified without limitation by complexing with proteins, polyadenylation, splicing, capping or export from the nucleus. The term "gene product" refers to any

20   product directly resulting from transcription of a gene. In particular this includes partial, precursor, and mature transcription products (i.e, pre-mRNA and mRNA), and translation products with or without further processing including, without limitation, lipidation, phosphorylation, glycosylation, or combinations of such processing

The term "gene involved in the origin or pathogenesis of a disease or condition"

25   refers to a gene that harbors mutations that contribute to the cause of disease, or variances that affect the progression of the disease or expression of specific characteristic of the disease. The term also applies to genes involved in the synthesis, accumulation, or elimination of products that are involved in the origin or pathogenesis of a disease or condition including, without limitation, proteins, lipids, carbohydrates, hormones, or

30   small molecules.

The term "gene involved in the action of a drug" refers to any gene whose gene product affects the efficacy or safety of the drug or affects the disease process being treated by the drug, and includes, without limitation, genes that encode gene products that

are targets for drug action, gene products that are involved in the metabolism, activation or degradation of the drug, gene products that are involved in the bioavailability or elimination of the drug to the target, gene products that affect biological pathways that, in turn, affect the action of the drug such as the synthesis or degradation of competitive

5   substrates or allosteric effectors or rate limiting reaction, or, alternatively, gene products that affect the pathophysiology of the disease process. (Particular variances in the latter category of genes may be associated with patient groups in whom disease etiology is more or less susceptible to amelioration by the drug. For example, there are several pathophysiological mechanisms in hypertension, and depending on the dominant

10   mechanism in a given patient, that patient may be more or less likely than the average hypertensive patient to respond to a drug that primarily targets one pathophysiological mechanism. The relative importance of different pathophysiological mechanisms in individual patients is likely to be affected by variances in genes associated with the disease pathophysiology. The "action" of a drug refers to its effect on biological products

15   within the body. The action of a drug also refers to its effects on the signs or symptoms of a disease or condition, or effects of the drug that are unrelated to the disease or condition leading to unanticipated effects on other processes. Such unanticipated processes often lead to adverse events or toxic effects. The terms "adverse event" or "toxic" event" are known in the art and include, without limitation, those listed in the

20   FDA reference system for adverse events.

In accordance with the aspects above and the Detailed Description below, there is also described for this invention an approach or method for developing drugs that are explicitly indicated for, and/or for which approved use is restricted to individuals in the population with specific variances or combinations of variances, as determined by

25   diagnostic tests for variances or variant forms of certain genes involved in the disease or condition or involved in the action of the drug. Such drugs may provide more effective treatment for a disease or condition in a population identified or characterized with the use of a diagnostic test for a specific variance or variant form of the gene if the gene is involved in the action of the drug or in determining a characteristic of the disease or

30   condition. Such drugs may be developed using the diagnostic tests for specific variances or variant forms of a gene to determine the inclusion of patients in a clinical trial.

Thus, the invention also provides a method for producing a pharmaceutical composition by identifying a compound which has differential activity against a disease

or condition in patients having at least one variance in a gene, compounding the pharmaceutical composition by combining the compound with a pharmaceutically acceptable carrier, excipient, or diluent such that the composition is preferentially effective in patients who have at least one copy of the variance or variances. In some

5   cases, the patient has two copies of the variance or variances. In preferred embodiments, the disease or condition, gene or genes, variances, methods of administration, or method of determining the presence or absence of variances is as described for other aspects of this invention.

Similarly, the invention provides a method for producing a pharmaceutical agent

10   by identifying a compound which has differential activity against a disease or condition in patients having at least one copy of a form of a gene having at least one variance and synthesizing the compound in an amount sufficient to provide a pharmaceutical effect in a patient suffering from the disease or condition. The compound can be identified by conventional screening methods and its activity confirmed. For example, compound

15   libraries can be screened to identify compounds which differentially bind to products of variant forms of a particular gene product, or which differentially affect expression of variant forms of the particular gene, or which differentially affect the activity of a product expressed from such gene. Preferred embodiments are as for the preceding aspect.

In another aspect, the invention provides a method of treating a disease or

20   condition in a patient by selecting a patient whose cells have an allele of a gene selected from the genes listed herein, preferably in Tables 2, 6, or 8. The allele contains at least one variance correlated with more effective response to a treatment of the disease or condition, or tolerance of a treatment, e.g., a treatment with a drug or a drug of a class indicated herein.

25   Preferably the allele contains a variance as shown in Tables 2, 6, or 8 or other variance table herein. Also preferably, the altering involves administering to the patient a compound preferentially active on at least one but less than all alleles of the gene. Preferred embodiments include those as described above for other aspects of treating a disease or condition.

30   In a further aspect, the invention provides a method for determining a method of treatment effective to treat a disease or condition by altering the level of activity of a product of an allele of a gene selected from the genes listed in Table 2, 6, or 8, and determining whether that alteration provides a differential effect related to reducing or

alleviating a disease or condition as compared to at least one alternative allele or an alteration in toxicity or tolerance of the treatment by a patient or patients. The presence of such a differential effect indicates that altering that level of activity provides at least part of an effective treatment for the disease or condition.

5   Preferably the determining is carried out in a clinical trial, e.g., as described above and/or in the Detailed Description below.

In still another aspect, the invention provides a method for evaluating differential efficacy of or tolerance to a treatment in a subset of patients who have a particular variance or variances in at least one gene by utilizing a clinical trial. In preferred

10   embodiments, the clinical trial is a Phase I, II, III, or IV trial. Preferred embodiments include the stratifications and/or analyses as described below in the Detailed Description.

In yet another aspect, the invention provides a method for identifying at least one variance in at least one gene using computer-based sequence analysis or variance scanning as known to those skilled in the art.

15   Preferably the at least one gene is a plurality of genes, preferably at least 10, 20, 50, 100, 200, 500, 1000, 5000, 10,000, or even more. Preferably sequence and/or variance information on the plurality of genes is acumulated in one database or a set of commonly accessible databases within a single local computer network or on a single computer.

20

In yet another aspect, the invention provides experimental methods for finding additional variances in any of the genes provided in the table of Table 2, 6, or 8. In addition to the sequence analysis method, a number of experimental methods can also beneficially be used to identify variances. Thus the invention provides methods for

25   producing cDNA (e.g., example 13) or genomic DNA and detecting additional variances in the genes provided in Table 2, 6, or 8 using the single strand conformation polymorphism (SSCP) method (Example 14), the T4 Endonuclease VII method (Example 15) or DNA sequencing (Example 16) or other methods pointed out below. The application of these methods to the identified genes will provide identification of

30   additional variances that can affect inter-individual variation in drug or other treatment response. One skilled in the art will recognize that many methods for experimental variance detection have been described (in addition to the exemplary methods of examples 14, 15 and 16) which can be utilized. These additional methods include

chemical cleavage of mismatches (see, e.g., Ellis TP, et al., Chemical cleavage of mismatch: a new look at an established method. *Human Mutation* 11(5):345-53, 1998), denaturing gradient gel electrophoresis (see, e.g., Van Orsouw NJ, et al., Design and application of 2-D DGGE-based gene mutational scanning tests. *Genet Anal.* 14(5-6):205-13, 1999) and heteroduplex analysis (see, e.g., Ganguly A, et al., Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA.* 90 (21):10325-9, 1993).

In embodiments any of the above methods involving determination of the presence or absence of a particular variance or variances, the method preferably involves determining the presence or absence using a cell sample from an individual or individuals. Thus, the methods can also involve obtaining a cell sample from an individual. The cell sample can be any of a variety of different cells, e.g., blood cells skin cells, muscle cells, normal cells, or cancer cells.

By "comprising" is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram showing the relationships of enzymes involved in 5-FU metabolism and inhibition of thymidylate formation. Enzymes: 1. uridine phosphorylase; 2. thymidine phosphorylase; 3. orotate phosphoribosyl transferase; 4. thymidine kinase; 5. uridine kinase; 6. ribonucletide reductase; 7. thymidylate synthase; 8. dCMP deaminase; 9. nucleoside monophosphate kinase; 10. nucleoside diphosphate kinase; 11. nucleoside diphosphatase or cytidylate kinase; 12: thymine phosphorylase. FH2 = dihydrofolate, FH4 = tetrahydrofolate. The Figure is adapted from Goodman & Gilman's The Pharmacological Basis of Therapeutics, ninth edition, McGraw Hill, 1996, p. 1249.

Figure 2 is a diagram showing the relationship of enzymes related to folate metabolism and formation of 5,10-methylenetetrahydrofolate. Enzymes: 1. Forminino-tetrahydrofolate cyclodeaminase; 2. methenyltetrahydrofolate synthetase; 3. methenyltetra-hydrofolate cyclohydrolase; 4. formyltetrahydrofolate synthetase; 5. formyltetrahydrofolate hydrolase; 6. formyltetrahydrofolate dehydrogenase; 7. methyleneltetrahydrofolate dehydrogenase; 8. methyleneltetrahydrofolate reductase (MTHFR); 9. homocysteine methyltransferase (also called methionine synthetase); 10. serine transhydroxymethylase; 11. glycine cleavage system; 12. thymidylate synthase; 13. dihydrofolate reductase. Abbreviations: THF = tetrahydrofolate; DHF = dihydrofolate. Note that THF appears twice (i.e. the product of step 6 is also substrate for enzymes 10 and 11. Step 12 also appears in Figure 1, above. This Figure is adapted from Mathews & van Holde, Biochemistry, The Benjamin/Cummings Publishing Co., Redwood City CA, 1990, page 697.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is generally described below in connection with cancer chemotherapy. However, the described approach and techniques are applicable to a variety of other treatments and to genes associated with the efficacy and safety of such other treatments, for example, genes function in the pathways identified below, along with the specific genes listed. The present invention identifies a number of genes in certain treatment-related pathways, and further identifies a number of genetic sequence variances in those genes. The present description further describes how to identify variances which correlate with variable treatment efficacy and further how to identify additional variances in the identified genes and how to determine the treatment response correlation of those additional variances.

Chemotherapy of cancer currently involves use of highly toxic drugs with narrow therapeutic indices. Although progress has been made in the

chemotherapeutic treatment of selected malignancies, most adult solid cancers remain highly refractory to treatment. Nonetheless, chemotherapy is the standard of care for most disseminated solid cancers. Chemotherapy often results in a significant fraction of treated patients suffering unpleasant or life-threatening side effects while

5   receiving little or no clinical benefit; other patients may suffer few side effects and/or have complete remission or even cure. Any test that could predict response to chemotherapy, even partially, would allow more selective use of toxic drugs, and could thereby significantly improve efficacy of oncologic drug use, with the potential to both reduce side effects and increase the fraction of responders.

10   Chemotherapy is also expensive, not just because the drugs are often costly, but also because administering highly toxic drugs requires close monitoring by carefully trained personnel, and because hospitalization is often required for treatment of (or monitoring for) toxic drug reactions. Information that would allow patients to be divided into likely responder vs. non-responder (or likely side effect) groups, with

15   only the former to receive treatment, would therefore also have a significant impact on the economics of cancer drug use.

Predicting Response to Chemotherapy

Several methods for predicting response to chemotherapy in individual
20   patients have been investigated over the years, ranging from the use of biochemical markers to testing drugs on a patient's cultured tumor cells. None of these methods has proven sufficiently informative and practical to gain wide acceptance. However, there are some specific examples of tests useful for predicting toxicity. For example, a diagnostic test to predict side effects associated with the antineoplastic drugs 6-
25   mercaptopurine, 6-thioguanine and azathioprine has begun to gain wide acceptance, particularly among pediatric oncologists. Severe toxicity of thiopurine drugs is associated with deficiency of the enzyme thiopurine methyltransferase (TPMT). Currently most TPMT testing is done using an enzyme assay, however the TPMT gene has been cloned and mutations associated with low TPMT levels have been
30   identified; genetic testing is beginning to supplant enzyme assays because genetic tests are more easily standardized and economical.

While there are no good tests that predict positive chemotherapeutic response, there is demonstrated utility to measuring estrogen and progesterone receptor levels in cancer tissue before selecting therapy directed at modulating
35   hormonal state. Measuring genetic variation in proteins that mediate the effects, course, outcome, and/or development of adverse events in those patients potentially receiving chemotherapy drugs is, in some respects, analogous to measuring ER and PR levels, which mediate the effects of hormones.

I.   **Outline: Identification of interpatient variation in response; identification of genes and variances relevant to drug action; development of diagnostic tests; and use of variance status to determine treatment**

5   Human therapeutic development follows a course from discovery and analysis in a laboratory (preclinical development) to testing the candidate therapeutic intervention in human subjects (clinical development). The preclinical development of candidate therapeutic interventions for use in the treatment of human disease, disorders, or conditions begins at the discovery stage whereby a candidate therapy is tested *in vitro* to

10   achieve a desired biochemical alteration of a biochemical or physiological event. If successful, the candidate is generally tested in animals to determine toxicity, adsorption, distribution, and metabolism within a living species. Occasionally, there are available animal models that mimic human diseases, disorders, and conditions in which testing the candidate therapeutic intervention can provide supportive data to warrant proceeding to

15   test the agent or compound in humans. When an agent or compound enters first in human studies, it is recognized that the prediction of whether the agent or product's preclinical success will be mimicked in humans is imperfect. Both safety and efficacy data will generally have to ultimately be determined in humans. Therefore, given economic constraints, and considering the complexities of human clinical trials, any technical

20   advance to assist those skilled in the art of drug development will be welcomed. Advances can be implemented by aiding identification of genetic markers associated with interpatient variation in response during preclinical development (thereby allowing development of non-allele selective agents), or by identification or optimization of clinical trial design parameters in order to achieve successful development of therapeutic

25   products at any stage of clinical development, or by identifying variables that will allow safe and efficacious use of a marketed product. Such advances will provide benefits in the form of therapeutic alternatives to those patients in need of medical care.

         As indicated in the Summary above, certain aspects of the present invention typically involve the following process, which need not occur separately or in the order

30   stated. Not all of these described processes must be present in a particular method, or need be performed by a single entity or organization or person. Additionally, if certain of the information is available from other sources, that information can be utilized in the present invention. The processes are as follows: a) variability between patients in the response to a particular treatment is observed; b) at least a portion of the variable

35   response is correlated with the presence or absence of at least one variance in at least one gene; c) an analytical or diagnostic test is provided to determine the presence or absence of the at least one variance in individual patients; d) the presence or absence of the

variance or variances is used to select a patient for a treatment or to select a treatment for a patient, or the variance information is used in other methods described herein.

A.   Identification of Interpatient Variability in Response to a Treatment

Interpatient variability is the rule, not the exception, in clinical therapeutics. One of the best sources of information on interpatient variability is the nurses and physicians supervising the clinical trial who accumulate a body of first hand observations of physiological responses to the drug in different normal subjects or patients. Evidence of interpatient variation in response can also be measured statistically, and may be best described by statistical measures that examine magnitude of response (beneficial or adverse) across a large number of subjects.

In accord with the other portions of this description, the present invention concerns DNA sequence variances that can affect one or more of:

    i.   The susceptibility of individuals to a disease;

    ii. The course or natural history of a disease;

    iii. The response of a patient with a disease to a medical intervention, such as, for example, a drug, a biologic substance, physical energy such as radiation therapy, or a specific dietary regimen. The ability to predict either beneficial or detrimental responses is medically useful.

Thus variation in any of these three parameters may constitute the basis for initiating a pharmacogenetic study directed to the identification of the genetic sources of interpatient variation. The effect of a DNA sequence variance or variances on disease susceptibility or natural history (i and ii, above) are of particular interest as the variances can be used to define patient subsets which behave differently in response to medical interventions such as those described in (iii).

In other words, a variance can be useful for customizing medical therapy at least for either of two reasons. First, the variance may be associated with a specific disease subset that behaves differently with respect to one or more therapeutic interventions (i and ii above); second, the variance may affect response to a specific therapeutic intervention (iii above). Consider for exemplary purposes pharmacological therapeutic interventions. In the first case, there may be no effect of a particular gene sequence variance on the observable pharmacological action of a drug, yet the disease subsets defined by the variance or variances differ in their response to the drug because, for example, the drug acts on a pathway that is more relevant to disease pathophysiology in one variance-defined patient subset than in another variance-defined patient subset. The second type of useful gene sequence variance affects the pharmacological action of a drug or other treatment. Effects on pharmacological responses fall generally into two categories; pharmacokinetic and pharmacodynamic effects. These effects have been

defined as follows in Goodman and Gilman's Phamacologic Basis of Therapeutics (ninth edition, McGraw Hill, New York, 1986): "Pharmacokinetics" deals with the absorption, distribution, biotransformations and excretion of drugs. The study of the biochemical and physiological effects of drugs and their mechanisms of action is termed

5   "pharmacodynamics."

Useful gene sequence variances for this invention can be described as variances which partition patients into two or more groups that respond differently to a therapy, regardless of the reason for the difference, and regardless of whether the reason for the difference is known.

10

B.   Identification of Specific Genes and Correlation of Variances in Those Genes with Response to Treatment of Diseases or Conditions

It is useful to identify particular genes which do or are likely to mediate the efficacy or safety of a treatment method for a disease or condition, particularly in view of

15   the large number of genes which have been identified and which continue to be identified in humans. As is further discussed in section C below, this correlation can proceed by different paths. One exemplary method utilizes prior information on the pharmacology or pharmacokinetics or pharmacodynamics of a treatment method, e.g., the action of a drug, which indicates that a particular gene is, or is likely to be, involved in the action of the

20   treatment method, and further suggests that variances in the gene may contribute to variable response to the treatment method.

Alternatively, if such information is not known, variances in a gene can be correlated empirically with treatment response. In this method, variances in a gene which exist in a population can be identified. The presence of the different variances or

25   haplotypes in individuals of a study group, which is preferably representative of a population or populations, is determined. This variance information is then correlated with treatment response of the various individuals as an indication that genetic variability in the gene is at least partially responsible for differential treatment response. Statistical measures known to those skilled in the art are preferably used to measure the fraction of

30   interpatient variation attributable to any one variance.

Useful methods for identifying genes relevant to the physiologic action of a drug or other treatment are known to those skilled in the art, and include large scale analysis of gene expression in cells treated with the drug compared to control cells, or large scale analysis of the protein expression pattern in treated vs. untreated cells, or the use of

35   techniques for identification of interacting proteins or ligand-protein interactions.

C.   Development of a Diagnostic Test to Determine Variance Status

In accordance with the description in the Summary above, the present invention generally concerns the identification of variances in genes which are indicative of the effectiveness of a treatment in a patient. The identification of specific variances, in effect, can be used as a diagnostic or prognostic test. Correlation of treatment efficacy
5   and/or toxicity with particular genes and gene families or pathways is provided in Stanton et al., U.S. Provisional Application 60/093,484, filed July 20, 1998, entitled GENE SEQUENCE VARIANCES WITH UTILITY IN DETERMINING THE TREATMENT OF DISEASE (concerns the safety and efficacy of compounds active on folate or pyrimidine metabolism or action).
10   Genes identified in the examples below and the attached Tables and Figures can be used in the present invention.

Methods for diagnostic tests are well known in the art. Generally in this invention, the diagnostic test involves determining whether an individual has a variance or variant form of a gene that is involved in the disease or condition or the action of the
15   drug or other treatment or effects of such treatment. Such a variance or variant form of the gene is preferably one of several different variances or forms of the gene that have been identified within the population and are known to be present at a certain frequency. In an exemplary method, the diagnostic test involves performed by amplifying a segment of DNA or RNA (generally after converting the RNA to cDNA) spanning one or more
20   variances in the gene sequence. Preferably, the amplified segment is <500 bases in length, in an alternative embodiment the amplified segment is <100 bases in length, most preferably <45 bases in length. In many cases, the diagnostic test is performed by amplifying a segment of DNA or RNA (cDNA) spanning a variance, or even spanning more than one variance in the gene sequence and preferably maintaining the phase of the
25   variances on each allele. The term "phase" means the association of variances on a single copy of the gene, such as the copy transmitted from the mother (maternal copy or maternal allele) or the father (paternal copy or paternal allele). It is apparent that such diagnostic tests are performed after initial identification of variances within the gene.

Diagnostic genetic tests useful for practicing this invention belong to two types:
30   genotyping tests and haplotyping tests. A genotyping test simply provides the status of a variance or variances in a subject or patient. For example suppose nucleotide 150 of hypothetical gene X on an autosomal chromosome is an adenine (A) or a guanine (G) base. The possible genotypes in any individual are AA, AG or GG at nucleotide 150 of gene X.

35   In a haplotyping test there is at least one additional variance in gene X, say at nucleotide 810, which varies in the population as cytosine (C) or thymine (T). Thus a particular copy of gene X may have any of the following combinations of nucleotides at positions 150 and 810: 150A-810C, 150A-810T, 150G-810C or 150G-810T. Each of the

four possibilities is a unique haplotype. If the two nucleotides interact in either RNA or protein, then knowing the haplotype can be important. The point of a haplotyping test is to determine the haplotypes present in a DNA or cDNA sample (e.g. from a patient). In the example provided there are only four possible haplotypes, but, depending on the

5   number of variances in the gene and their distribution in human populations there may be three, four, five, six or more haplotypes at a given gene. The most useful haplotypes for this invention are those which occur commonly in the population being treated for a disease or condition. Preferably such haplotypes occur in at least 5% of the population, more preferably in at least 10%, still more preferably in at least 20% of the population

10   and most preferably in at least 30% or more of the population. Conversely, when the goal of a pharmacogenetic program is to identify a relatively rare population that has an adverse reaction to a treatment, the most useful haplotypes may be rare haplotypes, which may occur in less than 5%, less than 2%, or even in less than 1% of the population. One skilled in the art will recognize that the frequency of the adverse reaction will provide a

15   useful guide to the likely frequency of salient causative haplotypes.

Based on the identification of variances or variant forms of a gene, a diagnostic test utilizing methods known in the art can be used to determine whether a particular form of the gene, containing specific variances or haplotypes, or combinations of variances and haplotypes, is present in at least one copy, one copy, or more than one copy in an

20   individual. Such tests are commonly performed using DNA or RNA collected from blood, cells, tissue scrapings or other cellular materials, and can be performed by a variety of methods including, but not limited to, hybridization with allele-specific probes, enzymatic mutation detection, chemical cleavage of mismatches, mass spectrometry or DNA sequencing, including minisequencing. Methods for haplotyping are provided in

25   this application. In particular embodiments, hybridization with allele specific probes can be conducted in two formats: (1) allele specific oligonucleotides bound to a solid phase (glass, silicon, nylon membranes) and the labelled sample in solution, as in many DNA chip applications, or (2) bound sample (often cloned DNA or PCR amplified DNA) and labelled oligonucleotides in solution (either allele specific or short so as to allow

30   sequencing by hybridization). The application of such diagnostic tests is possible after identification of variances that occur in the population. Diagnostic tests may involve a panel of variances from one or more genes, often on a solid support, which enables the simultaneous determination of more than one variance in one or more genes.

D.   Use of Variance Status to Determine Treatment

35   The present disclosure describes exemplary gene sequence variances in genes identified in a gene table herein (e.g., Tables 2, 6, and 8), and variant forms of these gene that may be determined using diagnostic tests. As indicated in the Summary, such a variance-based diagnostic test can be used to determine whether or not to administer a

specific drug or other treatment to a patient for treatment of a disease or condition. Preferably such diagnostic tests are incorporated in texts such as Clinical Diagnosis and Management by Laboratory Methods (19th Ed) by John B. Henry (Editor) W B Saunders Company, 1996; Clinical Laboratory Medicine : Clinical Application of Laboratory Data,

5   (6th edition) by R. Ravel, Mosby-Year Book, 1995, , or medical textbooks including, without limitation, textbooks of medicine, laboratory medicine, therapeutics, pharmacy, pharmacology, nutrition, allopathic, homeopathic, and osteopathic medicine; most preferably such a diagnostic test is specified by regulatory authorities, e.g., by the U.S. Food and Drug Administration, and is incorporated in the label or insert as well as the

10 Physicians Desk Reference.

In such cases, the procedure for using the drug is restricted or limited on the basis of a diagnostic test for determining the presence of a variance or variant form of a gene. The procedure may include the route of administration of the drug, the dosage form, dosage, schedule of administration or use with other drugs; any or all of these may

15 require selecting or determination consistent with the results of the diagnostic test or a plurality of such tests. Preferably the use of such diagnostic tests to determine the procedure for administration of a drug is incorporated in a text such as those listed above, or medical textbooks, for example, textbooks of medicine, laboratory medicine, therapeutics, pharmacy, pharmacology, nutrition, allopathic, homeopathic, and

20 osteopathic medicine. As previously stated, preferably such a diagnostic test or tests are required by regulatory authorities and are incorporated in the label or insert as well as the Physicians Desk Reference.

Variances and variant forms of genes useful in conjunction with treatment methods may be associated with the origin or the pathogenesis of a disease or condition.

25 In many useful cases, the variant form of the gene is associated with a specific characteristic of the disease or condition that is the target of a treatment, most preferably response to specific drugs or other treatments. Examples of diseases or conditions ameliorable by the methods of this invention are identified in the Examples and tables below; in general treatment of disease with current methods, particularly drug treatment,

30 always involves some unknown element (involving efficacy or toxicity or both) that can be reduced by appropriate diagnostic methods.

Alternatively, the gene is involved in drug action, and the variant forms of the gene are associated with variability in the action of the drug. For example, in some cases, one variant form of the gene is associated with the action of the drug such that the drug

35 will be effective in an individual who inherits one or two copies of that form of the gene. Alternatively, a variant form of the gene is associated with the action of the drug such that the drug will be toxic or otherwise contra-indicated in an individual who inherits one or two copies of that form of the gene.

In accord with this invention, diagnostic tests for variances and variant forms of genes as described above can be used in clinical trials to demonstrate the safety and efficacy of a drug in a specific population. As a result, in the case of drugs which show variability in patient response correlated with the presence or absence of a variance or

5 variances, it is preferable that such drug is approved for sale or use by regulatory agencies with the recommendation or requirement that a diagnostic test be performed for a specific variance or variant form of a gene which identifies specific populations in which the drug will be safe and/or effective. For example, the drug may be approved for sale or use by regulatory agencies with the specification that a diagnostic test be performed for a

10 specific variance or variant form of a gene which identifies specific populations in which the drug will be toxic. Thus, approved use of the drug, or the procedure for use of the drug, can be limited by a diagnostic test for such variances or variant forms of a gene; or such a diagnostic test may be considered good medical practice, but not absolutely required for use of the drug.

15 As indicated, diagnostic tests for variances as described in this invention may be used in clinical trials to establish the safety and efficacy of a drug. Methods for such clinical trials are described below and/or are known in the art and are described in standard textbooks. For example, diagnostic tests for a specific variance or variant form of a gene may be incorporated in the clinical trial protocol as inclusion or exclusion

20 criteria for enrollment in the trial, to allocate certain patients to treatment or control groups within the clinical trial or to assign patients to different treatment cohorts. Alternatively, diagnostic tests for specific variances may be performed on all patients within a clinical trial, and statistical analysis performed comparing and contrasting the efficacy or safety of a drug between individuals with different variances or variant forms

25 of the gene or genes. Preferred embodiments involving clinical trials include the genetic stratification strategies, phases, statistical analyses, sizes, and other parameters as described herein.

Similarly, diagnostic tests for variances can be performed on groups of patients known to have efficacious responses to the drug to identify differences in the frequency

30 of variances between responders and non-responders. Likewise, in other cases, diagnostic tests for variance are performed on groups of patients known to have toxic responses to the drug to identify differences in the frequency of the variance between those having adverse events and those not having adverse events. Such outlier analyses may be particularly useful if a limited number of patient samples are available for

35 analysis. It is apparent that such clinical trials can be or are performed after identifying specific variances or variant forms of the gene in the population.

The identification and confirmation of genetic variances is described in certain patents and patent applications. The description therein is useful in the identification of

variances in the present invention. For example, a strategy for the development of anticancer agents having a high therapeutic index is described in Housman, International Application PCT/US/94 08473 and Housman, INHIBITORS OF ALTERNATIVE ALLELES OF GENES ENCODING PROTEINS VITAL FOR CELL

5 VIABILITY OR CELL GROWTH AS A BASIS FOR CANCER THERAPEUTIC AGENTS, U.S. Patent 5,702,890, issued December 30, 1997, which are hereby incorporated by reference in their entireties. Also, a number of gene targets and associated variances are identified in Housman et al., U.S. Patent Application 09/045,053, entitled TARGET ALLELES FOR ALLELE-SPECIFIC DRUGS, filed

10 March 19, 1998, which is hereby incorporated by reference in its entirety, including drawings.

The described approach and techniques are applicable to a variety of other diseases, conditions, and/or treatments and to genes associated with the etiology and pathogenesis of such other diseases and conditions and the efficacy and safety of such

15 other treatments.

Useful variances for this invention can be described generally as variances which partition patients into two or more groups that respond differently to a therapy (a therapeutic intervention), regardless of the reason for the difference, and regardless of whether the reason for the difference is known.

20

II.   From Variance List to Clinical Trial: Identifying Genes and Gene Variances that Account for Variable Responses to Treatment

There are a variety of useful methods for identifying a subset of genes from a large set that should be prioritized for further investigation with respect to their influence

25 on inter-individual variation in disease predisposition or response to a particular drug. These methods include for example, (1) searching the relevant literature to identify genes relevant to a disease or the action of a drug; (2) screening the genes identified in step 1 for variances. A large set of exemplary variances are provided in Tables 3, 4, and 10; (3) using computational tools to predict the functional effects of variances in specific genes;

30 (4) using *in vitro* or *in vivo* experiments to identify genes which may participate in the response to a drug or treatment, and to determine the variances which affect gene, RNA or protein function, and may therefore be important genetic variables affecting disease manifestations or drug response; and (5) retrospective or prospective clinical trials. Each of these methods is considered below in some detail.

35

(1) To begin, one preferably identifies, for a given treatment, a set of candidate genes that are likely to affect disease phenotype or drug response. This can be accomplished most efficiently by first assembling the relevant medical, pharmacological and

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biological data from available sources (e.g., public databases and publications). One skilled in the art can review the literature (textbooks, monographs, journal articles) and online sources (databases) to identify genes most relevant to the action of a specific drug or other treatment, particularly with respect to its utility for treating a specific disease, as this beneficially allows the set of genes to be analyzed ultimately in clinical trials to be reduced from an initial large set. Specific strategies for conducting such searches are described below. In some instances the literature may provide adequate information to select genes to be studied in a clinical trial, but in other cases additional experimental investigations of the sort described below will be preferable to maximize the likelihood that the salient genes and variances are moved forward into clinical studies. Experimental data are also useful in establishing a list of candidate genes, as described below.

(2) Having assembled a list of candidate genes generally the second step is to screen for variances in each candidate gene. Experimental and computational methods for variance detection are described in this invention, and a tables of exemplary variances is provided (e.g., Table 3, 4, or 10) as well as methods for identifying additional variances.

(3) Having identified variances in candidate genes the next step is to assess their likely contribution to clinical variation in patient response to therapy, preferably by using informatics-based approaches such as DNA and protein sequence analysis and protein modeling. The literature and informatics-based approaches provide the basis for prioritization of candidate genes, however it may in some cases be desirable to further narrow the list of candidate genes, or to measure experimentally the phenotype associated with specific variances or sets of variances (e.g. haplotypes).

(4) Thus, as a third step in candidate gene analysis, one skilled in the art may elect to perform *in vitro* or *in vivo* experiments to assess the functional importance of gene variances, using either biochemical or genetic tests. (Certain kinds of experiments – for example gene expression profiling and proteome analysis - may not only allow refinement of a candidate gene list but may also lead to identification of additional candidate genes.) Combination of two or all of the three above methods will provide sufficient information to narrow the set of candidate genes and variances to a number that can be studied in a clinical trial with adequate statistical power.

(5) The fourth step is to design retrospective or prospective human clinical trials to test whether the identified allelic variance, variances, or haplotypes or combination thereof influence the efficacy or toxicity profiles for a given drug or other therapeutic intervention. It should be recognized that this fourth step is the crucial step in producing the type of data that would justify introducing a diagnostic test for at least one variance into clinical use. Thus while each of the above four steps are useful in

particular instances of the invention, this final step is indispensable. Further guidance and examples of how to perform these five steps is provided below.

1. Identification of Candidate Genes Relevant to the Action of a Drug

Practice of this invention will often begin with identification of a specific pharmaceutical product, for example a drug, that would benefit from improved efficacy or reduced toxicity or both, and the recognition that pharmacogenetic investigations as described herein provide a basis for achieving such improved characteristics. The question then becomes which of the genes and variances provided in this application, e.g., in Tables 3, 4, and 10, would be most relevant to interpatient variation in response to the drug. As discussed above, the set of relevant genes includes both genes involved in the disease process and genes involved in the interaction of the patient and the treatment – for example genes involved in pharmacokinetic and pharmacodynamic action of a drug. The biological and biomedical literature and online databases provide useful guidance in selecting such genes. Specific guidance in the use of these resources is provided below.

Review the literature and online sources

One way to find genes that affect response to a drug in a particular disease setting is to review the published literature and available online databases regarding the pathophysiology of the disease and the pharmacology of the drug. Literature or online sources can provide specific genes involved in the disease process or drug response, or describe biochemical pathways involving multiple genes, each of which may affect the disease process or drug response.

Alternatively, biochemical or pathological changes characteristic of the disease may be described; such information can be used by one skilled in the art to infer a set of genes that can account for the biochemical or pathologic changes. For example, to understand variation in response to a drug that modulates serotonin levels in a central nervous system (CNS) disorder associated with altered levels of serotonin one would preferably study, at a minimum, variances in genes responsible for serotonin biosynthesis, release from the cell, receptor binding, presynaptic reuptake, and degradation or metabolism. Genes responsible for each of these functions should be examined for variation that may account for interpatient differences in drug response or disease manifestations. As recognized by those skilled in the art, a comprehensive list of such genes can be obtained from textbooks, monographs and the literature.

There are several types of scientific information, described in some detail below, that are valuable for identifying a set of candidate genes to be investigated with respect to a specific disease and therapeutic intervention. First there is the medical literature, which provides basic information on disease pathophysiology and therapeutic interventions. A

subset of this literature is devoted to specific description of pathologic conditions. Second there is the pharmacology literature, which will provide additional information on the mechanism of action of a drug (pharmacodynamics) as well as its principal routes of metabolic transformation (pharmacokinetics) and the responsible proteins. Third there is
5 the biomedical literature (principally genetics, physiology, biochemistry and molecular biology), which provides more detailed information on metabolic pathways, protein structure and function and gene structure. Fourth, there are a variety of online databases that provide additional information on metabolic pathways, gene families, protein function and other subjects relevant to selecting a set of genes that are likely to affect the
10 response to a treatment.

Medical Literature

A good starting place for information on molecular pathophysiology of a specific disease is a general medical textbook such as Harrison's Principles of Internal Medicine,
15 14th edition, (2 Vol Set) by A.S. Fauci, E. Braunwald, K.J. Isselbacher, et al. (editors), McGraw Hill, 1997, or Cecil Textbook of Medicine (20th Ed) by R. L. Cecil, F. Plum and J. C. Bennett (Editors) W B Saunders Co., 1996. For pediatric diseases texts such as Nelson Textbook of Pediatrics (15th edition) by R.E. Behrman, R.M. Kliegman, A.M. Arvin and W.E. Nelson (Editors), W B Saunders Co., 1995 or Oski's Principles and
20 Practice of Pediatrics (3rd Edition) by J.A. Mamillan & F.A. Oski Lippincott-Raven, 1999 are useful introductions. For obstetrical and gynecological disorders texts such as Williams Obstetrics (20th Ed) by F.G. Cunningham, N.F. Gant, P.C. McDonald et al. (Editors), Appleton & Lange, 1997 provide general information on disease pathophysiology. For psychiatric disorders texts such as the Comprehensive Textbook of
25 Psychiatry, VI (2 Vols) by H.I. Kaplan and B.J. Sadock (Editors), Lippincott, Williams & Wilkins, 1995, or The American Psychiatric Press Textbook of Psychiatry (3rd edition) by R.E. Hales, S.C. Yudofsky and J.A. Talbott (Editors) Amer Psychiatric Press, 1999 provide an overview of disease nosology, pathophysiological mechanisms and treatment regimens.
30 In addition to these general texts, there are a variety of more specialized medical texts that provide greater detail about specific disorders which can be utilized in developing a list of candidate genes and variances relevant to interpatient variation in response to a treatment. For example, within the field of medicine there are standard textbooks for each of the subspecialties. Some specific examples include:
35 Heart Disease: A Textbook of Cardiovascular Medicine (2 Volume set) by E. Braunwald (Editor), W B Saunders Co., 1996.
Hurst's the Heart, Arteries and Veins (9th Ed) (2 Vol Set) by R.W. Alexander, R.C. Schlant, V. Fuster, W. Alexander and E.H. Sonnenblick (Editors) McGraw Hill,

1998.

Principles of Neurology (6th edition) by R.D. Adams, M. Victor (editors), and A.H. Ropper (Contributor), McGraw Hill, 1996.

Sleisenger & Fordtran's Gastrointestinal and Liver Disease: Pathophysiology, Diagnosis, Management (6th edition) by M. Feldman, B.F. Scharschmidt and M. Sleisenger (Editors), W B Saunders Co., 1997.

Textbook of Rheumatology (5th edition) by W.N. Kelley, S. Ruddy, E.D. Harris Jr. and C.B. Sledge (Editors) (2 volume set) W B Saunders Co., 1997.

Williams Textbook of Endocrinology (9th edition) by J.D. Wilson, D.W. Foster, H. M. Kronenberg and Larsen (Editors), W B Saunders Co., 1998.

Wintrobe's Clinical Hematology (10th Ed) by G.R. Lee, J. Foerster (Editor) and J. Lukens (Editors) (2 Volumes) Lippincott, Williams & Wilkins, 1998.

Cancer: Principles & Practice of Oncology (5th edition) by V.T. Devita, S.A. Rosenberg and S. Hellman (editors), Lippincott-Raven Publishers, 1997.

Principles of Pulmonary Medicine (3rd edition) by S.E. Weinberger & J Fletcher (Editors), W B Saunders Co., 1998.

Diagnosis and Management of Renal Disease and Hypertension (2nd edition) by A.K. Mandal & J.C. Jennette (Editors), Carolina Academic Press, 1994.Massry & Glassock's Textbook of Nephrology (3rd edition) by S.G. Massry & R.J. Glassock (editors) Williams & Wilkins, 1995.

The Management of Pain by J.J. Bonica, Lea and Febiger, 1992

Ophthalmology by M. Yanoff & J.S. Duker, Mosby Year Book, 1998

Clinical Ophthalmology: A Systemic Approach by J.J. Kanski, Butterworth-Heineman, 1994.Essential Otolaryngology by J.K. Lee Appleton and Lange 1998.

In addition to these subspecialty texts there are many textbooks and monographs that concern more restricted disease areas, or specific diseases. Such books provide more extensive coverage of pathophysiologic mechanisms and therapeutic options. The number of such books is too great to provide examples for all but a few diseases, however one skilled in the art will be able to readily identify relevant texts. One simple way to search for relevant titles is to use the search engine of an online bookseller such as http://www.amazon.com or http://www.barnesandnoble.com using the disease or drug (or the group of diseases or drugs to which they belong) as search terms. For example a search for asthma would turn up titles such as Asthma : Basic Mechanisms and Clinical Management (3rd edition) by P.J. Barnes, I.W. Rodger and N.C. Thomson (Editors), Academic Press, 1998 and Airways and Vascular Remodelling in Asthma and Cardiovascular Disease : Implications for Therapeutic Intervention : Based on the Scientific Program, by C. Page & J. Black (Editors), Academic Press, 1994.

Pathology Literature

In addition to medical texts there are texts that specifically address disease etiology and pathologic changes associated with disease. A good general pathology text is Robbins Pathologic Basis of Disease (6th edition) by R.S. Cotran, V. Kumar, T.

5 Collins and S.L. Robbins, W B Saunders Co., 1998. Specialized pathology texts exist for each organ system and for specific diseases, similar to medical texts. These texts are useful sources of information for one skilled in the art for developing lists of genes that may account for some of the known pathologic changes in disease tissue. Exemplary texts are as follows:

10 Bone Marrow Pathology 2nd edition, by B.J. Bain, I. Lampert. & D. Clark, Blackwell Science, 1996

Atlas of Renal Pathology by F.G. Silva, W.B. Saunders, 1999.

Fundamentals of Toxicologic Pathology by W.M. Haschek and C.G. Rousseaux, Academic Press, 1997.

15 Gastrointestinal Pathology by P. Chandrasoma, Appleton and Lange, 1998.

Ophthalmic Pathology with Clinical Correlations by J. Sassani, Lippincott-Raven, 1997.

Pathology of Bone and Joint Disorders by F. McCarthy, F.J. Frassica and A. Ross, W. B. Saunders, 1998.

Pulmonary Pathology by M.A. Grippi, Lippicott-Raven, 1995.

20 Neuropathology by D. Ellison, L. Chimelli, B. Harding, S. Love& J. Lowe, Mosby Year Book, 1997.

Greenfield's Neuropatholgy 6th edition by J.G. Greenfield, P.L. Lantos & D.I. Graham, Edward Arnold, 1997.

25 *Pharmacology, Pharmacogenetics and Pharmacy Literature*

There are also both general and specialized texts and monographs on pharmacology that provide data on pharmacokinetics and pharmacodynamics of drugs. The discussion of pharmacodynamics (mechanism of action of the drug)in such texts is often supported by a review of the biochemical pathway or pathways that are affected by

30 the drug. Also, proteins related to the target protein are often listed; it is important to account for variation in such proteins as the related proteins may be involved in drug pharmacology. For example, there are 14 known serotonin receptors. Various pharmacological serotonin agonists or antagonists have different affinities for these different receptors. Variation in a specific receptor may affect the pharmacology not only

35 of drugs intentionally targeted to that receptor, but also drugs targeted to different receptors, that may have differential action on two allelic forms of the non-targeted receptor. Thus genes encoding proteins structurally related to the target protein are useful for screening for variance in the present invention. A good general pharmacology text is

Goodman & Gilman's the Pharmacological Basis of Therapeutics (9th Ed) by J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon and A.G. Gilman (Editors) McGraw Hill, 1996. There are also texts that focus on the pharmacology of drugs for specific disease areas, or specific classes of drugs (e.g. natural products) or adverse drug

5   interactions, among other subjects. Specific examples include:

The American Psychiatric Press Textbook of Psychopharmacology (2nd edition) by A.F. Schatzberg & C.B. Nemeroff (Editors), Amer Psychiatric Press, 1998. ISBN: 0880488174

Essential Psychopharmacology : Neuroscientific Basis and Practical Applications

10   by N. Muntner and S.M. Stahl, Cambridge Univ Press, 1996.

There are also texts on pharmacogenetics which are particularly useful for identifying genes which may contribute to variable pharmacokinetic response. In addition there are texts on some of the major xenobiotic metabolizing proteins, such as the cytochrome P450 genes.

15   Pharmacogenetics of Drug Metabolism (International Encyclopedia of Pharmacology and Therapeutics) by Werner Kalow (Editor) Pergamon Press, 1992.

Genetic Factors in Drug Therapy : Clinical and Molecular Pharmacogenetics by D.A Price Evans, Cambridge Univ Press, 1993.

Pharmacogenetics (Oxford Monographs on Medical Genetics, 32) by W.W.

20   Weber, Oxford Univ Press, 1997.

Cytochrome P450 : Structure, Mechanism, and Biochemistry by P.R. Ortiz de Montellano (Editor), Plenum Publishing Corp, 1995.

Appleton & Lange's Review of Pharmacy, 6th edition, (Appleton & Lange's Review Series) by G.D. Hall & B.S. Reiss, Appleton & Lange, 1997.

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Genetics, Biochemistry and Molecular Biology Literature

In addition to the medical, pathology, and pharmacology texts listed above there are several information sources that one skilled in the art will turn to for information on the genetic, physiologic, biochemical, and molecular biological aspects of the disease,

30   disorder or condition or the effect of the therapeutic intervention on specific physiologic processes. The biomedical literature may include information on nonhuman organisms that is relevant to understanding the likely disease or pharmacological pathways in man.

Genetic texts may provide insight into the likely effect of an allelic variance, variances, or haplotypes on individual responses to a therapeutic intervention,

35   particularly if there are genetic variances known to effect drug response. Example 1 describes variances in the dihydropyrimidine dehydrogenase (DPD) gene locus and their effects on fluoropyrimidine catabolism. DPD is an example of a gene that, in rare mutant forms, is associated with severe fluoropyrimidine poisoning. It is reasonable to expect

that more common alleles may exist at the DPD locus and may affect fluoropyrimidine metabolism, thus accounting for interpatient variation. Thus the genetics of a rare allele or alleles may provide a basis for examining the effects of commonly occuring alleles on moderate phenotypes. The genetics of rare DPD deficiency is well described in medical

5 genetics textbooks listed below, for example see Scriver et al (full citation below).

Also provided below are illustrative texts which will aid in the identification of a pathway or pathways, and a gene or genes that may be relevant to interindividual variation in response to a therapy. Textbooks of biochemistry, genetics and physiology are often useful sources for such pathway information. In order to ascertain the

10 appropriate methods to analyze the effects of an alleleic variance, variances, or haplotypes in vitro, one skilled in the art will review existing information on molecular biology, cell biology, genetics, biochemistry; and physiology. Such texts are useful sources for general and specific information on the genetic and biochemical processes involved in disease and in drug action, as well as experimental procedures that may be

15 useful in performing in vitro research on an allelic variance, variances, or haplotye.

Texts on gene structure and function and RNA biochemistry will be useful in evaluating the consequences of variances that do not change the coding sequence. Such variances may alter the interaction of RNA with proteins or other regulatory molecules affecting RNA processing, polyadenylation, and export.

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Molecular and Cellular Biology
Molecular Cell Biology by H. Lodish, D. Baltimore, A. Berk, L. Zipurksy & J. Darnell, W H Freeman & Co., 1995.
"Essentials of Molecular Biology", D. Freifelder and MalacinskiJones and Bartlett, 1993.

25 "Genes and Genomes: A Changing Perspective", M. Singer and P. Berg, 1991. University Science Books
"Gene Structure and Expression", J.D. Hawkins, 1996. Cambridge University Press
Molecular Biology of the Cell, 2nd edition, B. Alberts et alGarland Publishing, 1994.,

30 *Molecular Genetics*
The Metabolic and Molecular Bases of Inherited Disease by C. R. Scriver, A.L. Beaudet, W.S. Sly (Editors), 7th edition, McGraw Hill, 1995
"Genetics and Molecular Biology", R. Schleif, 1994. 2nd edition, Johns Hopkins University Press

35 "Genetics", P.J. Russell, 1996. 4th edition, Harper Collins
"An Introduction to Genetic Analysis", Griffiths et al.1993. 5th edition, W.H. Freeman and Company
"Understanding Genetics: A molecular approach", Rothwell, 1993. Wiley-Liss

General Biochemistry

"Biochemistry", L. Stryer, 1995. W.H. Freeman and Company

"Biochemistry", D. Voet and J.G. Voet, 1995. John Wiley and Sons

5   "Principles of Biochemistry", A.L. Lehninger, D.L. Nelson, and M.M. Cox, 1993. Worth Publishers

"Biochemistry", G. Zubay, 1998. Wm. C. Brown Communications

"Biochemistry", C.K. Mathews and K.E. van Holde, 1990. Benjamin/Cummings

10   *Transcription*

"Eukaryotic Transcriptiuon Factors", D.S. Latchman, 1995. Academic Press

"Eukaryotic Gene Transcription", S. Goodbourn (ed.), 1996. Oxford University Press.

"Transcription Factors and DNA Replication", D.S. Pederson and N.H. Heintz, 1994. CRC Press/R.G. Landes Company

15   "Transcriptional Regulation", S.L. McKnight and K. Yamamoto (eds.), 1992. 2 volumes, Cold Spring Harbor Laboratory Press

RNA

"Control of Messenger RNA Stability", J. Belasco and G. Brawerman (eds.), 1993.

20   Academic Press

"RNA-Protein Interactions", Nagai and Mattaj (eds.), 1994. Oxford University Press

"mRNA Metabolism and Post-transcriptional Gene Regulation", Harford and Morris (eds.), 1997. Wiley-Liss

Translation

25   "Translational Control", J.W.B. Hershey, M.B. Mathews, and N. Sonenberg (eds.), 1995. Cold Spring Harbor Laboratory Press

General Physiology

"Textbook of Medical Physiology" 9th Edtion by A.C. Guyton and J.E. Hall W.B.

30   Saunders, 1997

"Review of Medical Physiology", 18th Edition by W.F. Ganong, Appleton and Lange, 1997

Online Databases

35   Those skilled in the art are familiar with how to search the literature, such as, e.g., libraries, online pubmed, abstract listings, and online mutation databases. One particularly useful resource is maintained at the web site of the National Center for Biotechnology Information (ncbi): http://www.ncbi.nlm.nih.gov/. From the ncbi site one

can access Online Mendelian Inheritance in Man (OMIM),. OMIM can be found at: http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html. OMIM is a medically oriented database of genetic information with entries for thousands of genes. The OMIM record number is provided for many of the genes in Table 10 (see column 3), and constitutes an excellent entry point for identification of references that point to the broader literature. Another useful site at NCBI is the Entrez browser, located at http://www3.ncbi.nlm.nih.gov/Entrez/. One can search genomes, polynucleotides, proteins, 3D structures, taxonomy or the biomedical literature (PubMed) via the Entrez site. More generally links to a number of useful sites with biomedical or genetic data are maintained at sites such as Med Web at the Emory University Health Sciences Center Library: http://WWW.MedWeb.Emory.Edu/MedWeb/; Riken, a Japanese web site at: http://www.rtc.riken.go.jp/othersite.html with links to DNA sequence, structural, molecular biology, bioinformatics, and other databases; at the Oak Ridge National Laboratory web site: http://www.ornl.gov/hgmis/links.html; or at the Yahoo website of Diseases and Conditions: http://dir.yahoo.com/health/diseases_and_conditions/index.html. Each of the indicated web sites has additional useful links to other sites.

Another type of database with utility in selecting the genes on a biochemical pathway that may affect the response to a drug are databases that provide information on biochemical pathways. Examples of such databases include the Kyoto Encyclopedia of Genes and Genomes (KEGG), which can be found at: http://www.genome.ad.jp/kegg/kegg.html. This site has pictures of many biochemical pathways, as well as links to other metabolic databases such as the well known Boehringer Mannheim biochemical pathways charts: http://www.expasy.ch/cgi-bin/search-biochem-index. The metabolic charts at the latter site are comprehensive, and excellent starting points for working out the salient enzymes on any given pathway.

Each of the web sites mentioned above has links to other useful web sites, which in turn can lead to additional sites with useful information.

Research Libraries

Those skilled in the art will often require information found only at large libraries. The National Library of Medicine (http://www.nlm.nih.gov/) is the largest medical library in the world and its catalogs can be searched online. Other libraries, such as university or medical school libraries are also useful to conduct searches. Biomedical books such as those referred to above can often be obtained from online bookstores as described above.

Biomedical Literature

To obtain up to date information on drugs and their mechanism of action and biotransformation; disease pathophysiology; biochemical pathways relevant to drug action and disease pathophysiology; and genes that encode proteins relevant to drug action and disease one skilled in the art will consult the biomedical literature . A widely

5   used, publically accessible web site for searching published journal articles is PubMed (http://www.ncbi.nlm.nih.gov/PubMed/). At this site, one can search for the most recent articles (within the last 1-2 months) or for specific details on methods that are less recent (back to 1966). Many Journals also have their own sites on the world wide web and can be searched online. For example see the IDEAL web site at:

10   http://www.apnet.com/www/ap/aboutid.html. This site is an online library, featuring full text journals from Academic Press and selected journals from W.B. Saunders and Churchill Livingstone. The site provides access (for a fee) to nearly 2000 scientific, technical, and medical journals.

15   *Experimental methods for identification of genes involved in the action of a drug*
There are a number of experimental methods for identifying genes and gene products that mediate or modulate the effects of a drug or other treatment. They encompass analyses of RNA and protein expression as well as methods for detecting protein – protein interactions and protein – ligand interactions. Two preferred

20   experimental methods for identification of genes that may be involved in the action of a drug are (1) methods for measuring the expression levels of many mRNA transcripts in cells or organisms treated with the drug (2) methods for measuring the expression levels of many proteins in cells or organisms treated with the drug.

RNA transcripts or proteins that are substantially increased or decreased in drug

25   treated cells or tissues relative to control cells or tissues are candidates for mediating the action of the drug. Other useful experimental methods include protein interaction methods such as the yeast two hybrid system and variants thereof which facilitate the detection of protein – protein interactions.

The pool of RNAs expressed in a cell is sometimes referred to as the

30   transcriptome. Methods for measuring the transcriptome, or some part of it, are known in the art. A recent collection of articles summarizing some current methods appeared as a supplement to the journal *Nature Genetics*. (The Chipping Forecast. Nature Genetics supplement, volume 21, January 1999.) Experiments have been described in model systems that demonstrate the utility of measuring changes in the transcriptome before

35   before and after changing the growth conditions of cells, for example by changing the nutritional status. The changes in gene expression help reveal the network of genes that mediate physiological responses to the altered growth condition. Similarly, the addition of a drug to the cellular or in vivo environment, followed by monitoring the changes in

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gene expression can aid in identification of pharmacological gene networks.

The pool of proteins expressed in a cell is sometimes referred to as the proteome. Studies of the proteome may include not only protein abundance but also protein subcellular localization and protein-protein interaction. Methods for measuring the
5   proteome, or some part of it, are known in the art. One widely used method is to extract total cellular protein and separate it in two dimensions, for example first by size and then by isoelectric point. The resulting protein spots can be stained and quantitated, and individual spots can be excised and analyzed by mass spectrometry to provide definitive identification. The results can be compared from two or more cell lines or tissues, at least
10   one of which has been treated with a drug. The differential up or down modulation of specific proteins in response to drug treatment may indicate their role in mediating the pharmacologic actions of the drug. Another way to identify the network of proteins that mediate the actions of a drug is to exploit methods for identifying interacting proteins. By starting with a protein known to be involved in the action of a drug – for example the
15   drug target – one can use systems such as the yeast two hybrid system and variants thereof (known to those skilled in the art) to identify additional proteins in the network of proteins that mediate drug action. The genes encoding such proteins would be useful for screening for DNA sequence variances, which in turn may be useful for analysis of interpatient variation in response to treatments. For example, the protein 5-lipoxygenase
20   (5LO) s an enzyme which is a the beginning of the leukotriene biosynthetic pathway and is a target for anti-inflammatory drugs used to treat asthma and other diseases. In order to detect proteins that interact with 5-lipoxygenase the two-hybrid system was recently used to isolate three different proteins, none previously known to interact with 5LO. (Provost et al., Interaction of 5-lipoxygenase with cellular proteins. Proc. Natl. Acad. Sci. U.S.A.
25   96: 1881-1885, 1999.) A recent collection of articles summarizing some current methods in proteomics appeared in the August 1998 issue of the journal Electrophoresis (volume 19, number 11). Other useful articles include: Blackstock WP, et al. Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotechnol. 17 (3): p. 121-7, 1999, and Patton W.F., Proteome analysis II. Protein subcellular redistribution: linking
30   physiology to genomics via the proteome and separation technologies involved. J Chromatogr B Biomed Sci App. 722(1-2):203-23. 1999.

Since many of these methods can also be used to assess whether specific polymorphisms are likely to have biological effects, they should also be considered as relevant in section 3, below, concerning methods for assessing the likely contribution of
35   variances in candidate genes to clinical variation in patient responses to therapy.

2. Screen for Variances in Genes that may be Related to Therapeutic Response

Having identified a set of genes that may affect response to a drug the next step is to screen the genes for variances that may account for interindividual variation in

response to the drug. There are a variety of levels at which a gene can be screened for variances, and a variety of methods for variance screening. The two main levels of variance screening are genomic DNA screening and cDNA screening. Genomic variance detection may include screening the entire genomic segment spanning the gene from the

5   transcription start site to the polyadenylation site. Alternatively genomic variance detection may (for intron containing genes) include the exons and some region around them containing the splicing signals, for example, but not all of the intronic sequences. In addition to screening introns and exons for variances it is generally desirable to screen regulatory DNA sequences for variances. Promoter, enhancer, silencer and other

10   regulatory elements have been described in human genes. The promoter is generally proximal to the transcription start site, although there may be several promoters and several transcription start sites. Enhancer, silencer and other regulatory elements may be intragenic or may lie outside the introns and exons, possibly at a considerable distance, such as 100 kb away. Variances in such sequences may affect basal gene expression or

15   regulation of gene expression. In either case such variation may affect the response of an individual patient to a therapeutic intervention, for example a drug, as described in the examples. Thus in practicing the present invention it is useful to screen regulatory sequences as well as transcribed sequences, in order to identify variances that may affect gene transcription. Frequently information on the genomic sequence of a gene can be

20   found in the sources above, particularly by searching GenBank or Medline (PubMed). The name of the gene can be entered at a site such as Entrez: http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html. Using the genomic sequence and information from the biomedical literature one skilled in the art can perform a variance detection procedure such as those described in examples 14, 15 and 16.

25   Variance detection is often first performed on the cDNA of a gene for several reasons. First, available data on functional sequence variances suggests that variances in the transcribed portion of a gene are most likely to have functional consequences as they can affect the interaction of the transcript with a wide variety of cellular factors during the complex processes of transcription, processing and translation. Second, as a practical

30   matter the cDNA sequence of a gene is often available before the genomic structure is known, although the reverse may be true in the future as the sequence of the human genome is determined. If the genomic structure is not known then only the cDNA seqence can be scanned for variances. Methods for preparing cDNA are described in Example 13. Methods for variance detection on cDNA are described below and in the

35   examples.

          Methods for variance screening have been described, including DNA sequencing. See for example: US5698400: Detection of mutation by resolvase cleavage; US5217863: Detection of mutations in nucleic acids; and US5750335: Screening for genetic variation,

as well as the examples and references cited therein for examples of useful variance detection procedures. Detailed variance detection procedures are also described in examples 14, 15 and 16. One skilled in the art will recognize that depending on the specific aims of a variance detection project (number of genes being screened, number of individuals being screened, total length of DNA being screened) one of the above cited methods may be preferable to the others, or yet another procedure may be optimal. A preferred method of variance detection is chain terminating DNA sequencing using dye labeled primers, cycle sequencing and software for assessing the quality of the DNA sequence as well as specialized software for calling heterozygotes. The use of such procedures has been described by Nickerson and colleagues. See for example: Rieder M.J., et al. Automating the identification of DNA variations using quality-based fluorescence re-sequencing: analysis of the human mitochondrial genome. *Nucleic Acids Res.* 26 (4):967-73, 1998, and: Nickerson D.A., et al. PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res.* 25 (14):2745-51, 1997.Although the variances provided in tables 3, 4, and 10 consist principally of cDNA variances, it is a part of this invention that detection of genomic variances is also a useful method for identification of variances that may account for interpatient variation in response to a therapy.

3. Assess the Likely Contribution of Variances in Candidate Genes to Clinical Variation in Patient Responses to Therapy

Once a set of genes likely to affect disease pathophysiology or drug action has been identified, and those genes have been screened for variances, said variances (e.g., provided in Tables 3, 4, and 10) can be assessed for their contribution to variation in the pharmacological or toxicological phenotypes of interest. There are several methods which can be used in the present invention for assessing the medical and pharmaceutical implications of a DNA sequence variance. They range from computational methods to *in vitro* and/or *in vivo* experimental methods (discussed below), to prospective human clinical trials (see below), and also include a variety of other laboratory and clinical measures that can provide evidence of the medical consequences of a variance. In general, human clinical trials constitute the highest standard of proof that a variance or set of variances is useful for selecting a method of treatment, however, computational and *in vitro* data, or retrospective analysis of human clinical data may provide strong evidence that a particular variance will affect response to a given therapy. Moreover, at an early stage in the analysis when there are many possible hypotheses to explain interpatient variation in treatment response, the use of informatics-based approaches to evaluate the likely functional effects of specific variances is an efficient way to proceed.

Informatics-based approaches to the prediction of the likely functional effects of variances include DNA and protein sequence analysis (phylogenetic approaches and motif searching) and protein modeling (based on coordinates in the protein database, or pdb; see http://www.rcsb.org/pdb/). Such analyses can be performed quickly and
5 inexpensively, and the results allow selection of certain genes for more extensive *in vitro* or *in vivo* studies (see below) or for more variance detection (see above) or both.

More specifically, the structure of many medically and pharmaceutically important proteins, or homologs of such proteins in other species, or examples of domains present in such proteins, is known. Further, there are increasingly powerful
10 tools for modeling the structure of proteins with unsolved structure, particularly if there is a related (e.g., a homologous) protein with known structure. (For reviews see: Rost et al., Protein fold recognition by prediction-based threading, *J. Mol. Biol.* 270:471-480, 1997; Firestine et al., Threading your way to protein function, *Chem. Biol.* 3:779-783, 1996) There are also powerful methods for identifying conserved domains and vital amino acid
15 residues of proteins of unknown structure by analysis of phylogenetic relationships. (Deleage et al., Protein structure prediction: Implications for the biologist, *Biochimie* 79:681-686, 1997; Taylor et al., Multiple protein structure alignment, *Protein Sci.* 3:1858-1870, 1994) These methods can permit the prediction of functionally important variances, either on the basis of structure or evolutionary conservation. For example, a
20 crystal structure can reveal which amino acids comprise a small molecule binding site. The identification of a polymorphic amino acid variance in the topological neighborhood of such a site, and in particular, the demonstration that at least one variant form of the protein has a variant amino acid which impinges on the known small molecule binding pocket differently from another variant form, provides strong evidence that the variance
25 affects the function of the protein. From this it follows that the interaction of the protein with a treatment method, such an administered drug, will also likely be altered. One skilled in the art will recognize that the application of computational tools to the identification of functionally consequential variances involves applying the knowledge and tools of medicinal chemistry and physiology to the analysis.
30

Phylogenetic approaches to understanding sequence variation are also useful. Thus if a sequence variance occurs at a nucleotide or encoded amino acid residue where there is usually little or no variation in homologs of the protein of interest from non-human species, particularly evolutionarily remote species, then the variance is more
35 likely to affect function of the RNA or protein.

4. Perform *in vitro* or *in vivo* Experiments to Assess the Functional Importance of Gene Variances

The selection of an appropriate experimental program for testing the medical consequences of a variance may differ depending on the nature of the variance, the gene, and the disease. For example if there is already evidence that a protein is involved in the pharmacologic action of a drug, then the *in vitro* demonstration that an amino acid

5   variance in the protein affects its biochemical activity is strong evidence that the variance will have an effect on the pharmacology of the drug in patients, and therefore that patients with different variant forms of the gene may have different responses to the same dose of drug. If the variance is silent with respect to protein coding information, or if it lies in a noncoding portion of the gene (e.g., a promoter, an intron, or a 5'- or 3'-untranslated

10 region) then the appropriate biochemical assay may be to assess mRNA abundance, half life, or translational efficiency. If, on the other hand, there is no substantial evidence that the protein encoded by a particular gene is relevant to drug pharmacology, then the appropriate test is a clinical study addressing the responses to therapy of two patient groups distinguished on the basis of one or more variances. This approach reflects the

15 current reality that biologists do not sufficiently understand gene regulation and gene expression to consistently make accurate inferences about the consequences of DNA sequence variances.

Thus, if there is a reasonable hypothesis regarding the effect of a protein on the action of a drug, then the *in vitro* and *in vivo* approaches described below will usefully

20 predict whether a given variance is therapeutically consequential. If, on the other hand, there is no evidence of such an effect, then the most appropriate test is the empirical clinical measure of efficacy (which requires no evidence or assumptions regarding the mechanism by which the variance may exert an effect on a therapy). Clinical studies may be performed either prospectively or retrospectively.

25

Experimental Methods: Genomic DNA Analysis

Variances in DNA may affect the basal transcription or regulated transcription of a gene locus. Such variances may be located in any part of the gene but are most likely to be located in the promoter region, the first intron, or in 5' or 3' flanking DNA, where

30 enhancer or silencer elements may be located. Methods for analyzing transcription are well known to those skilled in the art and exemplary methods are described in some of the texts cited below. Transcriptional run off assay is one useful method. Detailed protocols for useful methods can be found in texts such as: <u>Current Protocols in Molecular Biology</u> edited by: F.M. Ausubel, R.Brent, R.E. Kingston, D.D. Moore, J.G.

35 Seidman, K. Struhl, John Wiley & Sons, Inc, 1999, or: Molecular Cloning: A Laboratory Manual by J. Sambrook, E.F. Fritsch and T Maniatis. 1989. 3 vols, 2nd edition, Cold Spring Harbor Laboratory Press

Experimental Methods: RNA Analysis

RNA variances may affect a wide range of processes including RNA splicing, polyadenylation, capping, export from the nucleus, interaction with translation intiation, elongation or termination factors, or the ribosome, or interaction with cellular factors including regulatory proteins, or factors that may affect mRNA half life. However, any effect of variances on RNA function should ultimately be measurable as an effect on RNA levels – either basal levels or regulated levels or levels in some abnormal cell state. Therefore one preferred method for assessing the effect of RNA variances on RNA function is to measure the levels of RNA produced by different alleles in one or more conditions of cell or tissue growth. Said measuring can be done by conventional methods such as Northern blots or RNAase protection assays (kits available from Ambion, Inc.), or by methods such as the Taqman assay (developed by the Applied Biosystems Division of the Perkin Elmer Corporation), or by using arrays of oligonucleotides or arrays of cDNAs attached to solid surfaces. Systems for arraying cDNAs are available commercially from companies such as Nanogen and General Scanning. Complete systems for gene expression analysis are available from companies such as Molecular Dynamics. For recent reviews of the technology see the supplement to volume 21 of Nature Genetics entitled "The Chipping Forecast", especially articles beginning on pages 9, 15, 20 and 25.

Additional methods for analyzing the effect of variances on RNA include secondary structure probing, and direct measurement of half life or turnover. Secondary structure can be determined by techniques such as enzymatic probing (using enzymes such as T1, T2 and S1 nuclease), chemical probing or RNAase H probing using oligonucleotides. Some RNA structural assays can be performed *in vitro* or on cell extracts or on

Experimental Methods: Protein Analysis

There are a variety of experimental methods for investigating the effect of a variance on response of a patient to a treatment. The preferred method will depend on the availability of cells expressing a particular protein, and the feasibility of a cell-based assay vs. assays on cell extracts, on proteins produced in a foreign host, or on proteins prepared by *in vitro* translation.

For example, the methods and systems listed below can be utilized to demonstrate differential expression and/or activity, or in model system phenotype/genotype correlations.

For the determination of protein levels or protein activity one could utilize a variety of techniques. The in vitro protein activity can be determined by transcription or translation in bacteria, yeast, baculovirus, COS cells (transient), CHO, or study directly in

human cells. Further, one could perform pulse chase for experiments for the determination of changes in protein stability (half life).

One skilled in the art could manipulate the cell assay to address grouping the cells by genotypes or phenotypes. For example, identification of cells with different genotypes (possibly including families) and phenotype may be performed using standardized laboratory molecular biological protocols. After identification and grouping, one skilled in the art could determine whether there exists a correlation between cellular genotype and cellular phenotype.

Advancing an experimental preclinical program may include testing these in vitro hypotheses in vivo, e.g. an animal model. For example, one skilled in the art would readily have the ability to create gene knockouts. In this case, an embryonic stem cell is genetically manipulated to be deficient in a given gene. More specifically, a DNA construct is created that will undergo homologous recombination when inserted into the said embryonic stem cell nucleus. After the recombination event has occurred, the targeted gene is effectively inactivated due to the insertion of sequence (usually a translation stop or a marker gene sequence). This can be accomplished in worms, drosophila, or mice. The species chosen will be conducive to attain maximal experimental results for the particular gene and the particular variance, variances, or haplotype. Once the knockout species is created the candidate therapeutic intervention can be administered to the animal and tested for effects on gene expression or effects of various gene deficiencies. In the case whereby the chosen cell is a lower eukaryote, e.g. yeast, genetic manipulation occurs via introduction of a DNA construct that will undergo homologous recombination to disrupt the endogenous gene or genes.

The methods described above are reviewed and compiled in the following list of texts.

General Molecular Biology Methods

"Molecular Biology: A project approach", S.J. Karcher, Fall 1995. Academic Press

"DNA Cloning: A Practical Approach", D.M. Glover and B.D. Hayes (eds). 1995. IRL/Oxford University Press. Vol. 1 - Core Techniques; Vol 2 - Expression Systems; Vol. 3 - Complex Genomes; Vol. 4 -Mammalian Systems.

"Short Protocols in Molecular Biology", Ausubel et al. October 1995. 3rd edition, John Wiley and Sons

Current Protocols in Molecular Biology Edited by: F.M. Ausubel, R.Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, K. Struhl, (Series Editior: V.B. Chanda), 1988

"Molecular Cloning: A laboratory manual", J. Sambrook, E.F. Fritsch. 1989. 3 vols, 2nd edition, Cold Spring Harbor Laboratory Press

Polymerase chain reaction (PCR)

"PCR Primer: A laboratory manual", C.W. Diffenbach and G.S. Dveksler (eds.), 1995. Cold Spring Harbor Laboratory Press

"The Polymerase Chain Reaction", K.B. Mullis et al. (eds.), 1994. Birkhauser

"PCR Strategies", M.A. Innis, D.H. Gelf, and J.J. Sninsky (eds.), 1995. Academic Press

General procedures for discipline specific studies

Current Protocols in Neuroscience Edited by: J. Crawley, C. Gerfen, R. McKay, M. Rogawski, D. Sibley, P. Skolnick, (Series Editor: G. Taylor), 1997

Current Protocols in Pharmacology Edited by: S. J. Enna / M. Williams, J.W. Ferkany, T. Kenakin, R.E. Porsolt, J.P. Sullivan, (Series Editor: G. Taylor),1998

Current Protocols in Protein Science Edited by: J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, P.T. Wingfield, (Series Editor: Virginia Benson Chanda), 1995

Current Protocols in Cell Biology Edited by: J.S. Bonifacino, M. Dasso, J. Lippincott-Schwartz, J.B. Harford, K.M. Yamada, (Series Editor: K. Morgan) 1999

Current Protocols in Cytometry Managing Editor: J.P. Robinson, Z. Darzynkiewicz (ed) / P. Dean (ed), A. Orfao (ed), P. Rabinovitch (ed), C. Stewart (ed), H. Tanke (ed), L. Wheeless (ed), (Series Editor: J. Paul Robinson), 1997

Current Protocols in Human Genetics Edited by: N.C. Dracopoli, J.L. Haines, B.R. Korf, D.T. Moir, C.C. Morton, C.E. Seidman, J.G. Seidman, D.R. Smith, (Series Editor: A. Boyle), 1994

Current Protocols in Immunology Edited by: J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, (Series Editor: R. Coico), 1991

III. Clinical Trials

A clinical trial is the definitive test of the utility of a variance or variances for the selection of optimal therapy. Clinical trials require no knowledge of the biological function of the gene containing the variance or variances to be assessed, nor any knowledge of how the therapeutic intervention to be assessed works at a biochemical level; the question of the utility of a variance can be addressed at a purely phenomenological level. On the other hand, if there is information about either the biochemical basis of a therapeutic intervention or the biochemical effects of a variance, then a clinical trial can be designed to test a specific hypothesis.

Methods for performing clinical trials are well known in the art. (Guide to Clinical Trials by Bert Spilker, Raven Press, 1991; The Randomized Clinical Trial and

Therapeutic Decisions by Niels Tygstrup (Editor), Marcel Dekker; Recent Advances in Clinical Trial Design and Analysis (Cancer Treatment and Research, Ctar 75) by Peter F. Thall (Editor) Kluwer Academic Pub, 1995. However, performing a clinical trial to test the genetic contribution to interpatient variation in drug response requires some

5   additional design considerations, including defining what the genetic hypothesis is, how it is to be tested, how many patients will need to be enrolled to have adequate statistical power to measure an effect of a specified magnitude (power analysis), definition of primary and secondary endpoints, and methods of statistical analysis, as well as other aspects. In the outline below some of the major types of genetic hypothesis testing,

10 power analysis, statistical analysis, etc. are summarized. One skilled in the art will recognize that certain of the methods will be best suited to specific clinical situations, and that additional methods are known and can be used in particular instances.

A. *Performing a Clinical Trial*

15   As used herein, a "clinical trial" is the testing of a therapeutic intervention in a volunteer human population for the purpose of determining whether a therapeutic intervention is safe and/or efficacious in the human volunteer or patient population for a given disease, disorder, or condition. The analysis of safety and efficacy in genetically defined subgroups differing by at least one variance is of particular interest.

20   A "clinical study" is that part of a clinical trial that involves determination of the effect a candidate therapeutic intervention on human subjects. It includes clinical evaluations of physiologic responses including pharmacokinetic (absorption, distribution, bioavailability, and excretion) as well as pharmacodynamic (physiologic response and efficacy) parameters. A pharmacogenetic clinical study is a clinical study that involves

25 testing of one or more specific hypotheses regarding the effect of a genetic variance or variances (or set of variances, i.e. haplotype or haplotypes) in enrolled subjects or patients on response to a therapeutic intervention. These hypotheses are articulated before the study in the form of primary or secondary endpoints. For example the endpoint may be that in a particular genetic subgroup the rate of objectively defined responses exceeds

30 some predefined threshold.

        For each clinical study to commence enrollment and proceed to treat subjects at a given institution, an application that describes in detail the scientific premise for the therapeutic intervention and the procedures involved in the study, including the endpoints and analytical methods to be used in evaluating the data must be reviewed and accepted

35 by regulatory authorities at the level of the institution and the federal government (in the U.S.). In the U.S., there are two regulatory bodies that oversee conduct of clinical trials: an Institutional Review Board (IRB) and the United States Food and Drug Administration (US FDA). The European counterpart of the US FDA is the European Medicines

Evaluation Agency (EMEA). Similar agencies exist in other countries.

An Institutional Review Board accepts and reviews applications for clinical trials that are to be conducted at the institution and are to include healthy volunteers or human subjects from a defined patient population that seeks medical, surgical, rehabilitative, or

5   social services at that institution. The application includes document sections that provide the rationale for and describe the scope of the clinical study. For example, an application to an IRB may include a clinical protocol, and informed consent forms.

It is also customary, but not required, to prepare an investigator's brochure which describes the scientific hypothesis for the proposed therapeutic intervention, the

10   preclinical data, and the clinical protocol in concise language. The brochure is made available to any physician participating in the proposed or ongoing trial. The investigator's brochure for a pharmacogenetic clinical trial will include a full description of the genetic variance and/or variances believed or hypothesized to account for differential responses in the normal human subjects or patients, as well as a description of

15   the genetic statistical analysis.

The supporting preclinical data is a report of all the *in vitro, in vivo* animal or previous human trial data that supports the safety and/or efficacy of a given therapeutic   ---
intervention. In a pharmacogenetic clinical trial the preclinical data may also include a description of the effect of a specific genetic variance or variances on biochemical or

20   physiologic experimental variables, or on treatment outcomes, as determined by *in vitro* studies or by retrospective genetic analysis of clinical trial or other medical data (see below) used to first formulate or test a pharmacogenetic hypothesis.

The clinical protocol provides the relevant scientific and therapeutic introductory information, describes the inclusion and exclusion criteria for human subject enrollment,

25   including genetic criteria if relevant, describes in detail the exact procedure or procedures for treatment using the candidate therapeutic intervention, describes laboratory analyses to be performed during the study period, and lastly describes the risks (both known and unknown) involving the use of the experimental candidate therapeutic intervention. In a clinical protocol for a pharmacogenetic clinical trial, the clinical protocol will further

30   describe the gene or genes believed or hypothesized to affect differential patient responses and the variance or variances to be tested. Further, the clinical protocol for a pharmacogenetic clinical trial will include a description of the stratification of the treatment groups based on one or more gene sequence variances or combination of variances or haplotypes.

35   The informed consent document is a description of the therapeutic intervention and the clinical protocol in simple language (third grade level) for the patient to read, understand, and, if willing, agree to participate in the study by signing the document. In a pharmacogenetic clinical study the informed consent document will describe, in simple

language, the use of a genetic test or a limited set of genetic tests to determine the subject or patients status at a particular gene variance or variances, and to further ascertain whether, in the study population, particular variances are associated with particular clinical or physiological responses.

5   The US FDA reviews proposed clinical trials through the process of an Investigational New Drug Application (IND). The IND is composed of the investigator's brochure, the supporting *in vitro* and *in vivo* animal or previous human data, the clinical protocol, and the informed consent documents or forms. In each of the sections of the IND, a specific description of a single allelic variance or a number of variances to be

10   tested in the clinical study will be included. For example, in the investigator's brochure a description of the gene or genes believed or hypothesized to account, at least in part, for differential responses will be included as well as a description of genetic variance or variances of a particular candidate gene or genes. Further, the preclinical data may include a description of *in vivo* or *in vitro* studies of the biochemical or physiologic

15   effects of a variance or variances (e.g., haplotype) in a candidate gene or genes, as well as the predicted effects of the variance or variances on efficacy or toxicology of the candidate therapeutic intervention. Alternatively the results of retrospective genetic analysis of response data in patients treated with the candidate therapy may be the basis for formulating the genetic hypotheses to be tested in the prospective trial. For first in

20   man clinical studies, the focus of this section will be safety. The US FDA reviews the application with a particular emphasis on the safety data and whether toxicological data is supportive and sufficient to justify proceeding to human testing.

The established phases of clinical development are Phase I, II, III, and IV. The fundamental objectives for each phase become increasingly complex as the stages of

25   clinical development progress. In Phase I, safety in humans is the primary focus. In these studies, dose-ranging designs establish whether the candidate therapeutic intervention is safe in the suspected therapeutic concentration range. In a pharmacogenetic clinical trial there may be an analysis of the effect of a variance or variances on Phase I safety or surrogate efficacy parameters. At the same time,

30   pharmacokinetic parameters (e.g., adsorption, distribution, metabolism, and excretion) may be a secondary objective. In a pharmacogenetic clinical study, there may be additional analysis of the gene or genes and allelic variance or variances that are suspected to be involved in these pharmacokinetic parameters. As clinical development stages progress, trial objectives focus on the appropriate dose to elicit a therapeutically

35   relevant response. In a pharmacogenetic clinical trial, the dose or doses selected may be different than those identified based upon preclinical safety and efficacy determinations. For example, phenotypic effects of an allele depends on its frequency and also its interaction with the environment, as described earlier. Therefore, once the frequency of

an allele or haplotype has been established for selected human subjects or patients, the effect of the variance on the drug responses by performing both in vitro or in vivo analyses under controlled conditions. Under these conditions, drug dosage could be adjusted accordingly. In some instances, the chosen dose may be one that is sub-optimal

5   or is significantly less toxic so that determination of the effect of allelic variance or variances for a given treatment or human volunteer population may be appropriately tested and analyzed. In other instances, the dose may be similar to or the same as that chosen based upon *in vitro* or *in vivo* data. In yet other instances, the dose may be greater than optimal because allelic differences or haplotypes may result in enhanced elimination,

10 metabolic inactivation, or excretion.

Lastly, the objectives in the latter stages of clinical development center on the effect of the therapeutic intervention on the general population. In these trials, the numbers of individuals required for enrollment and the number of treatment conditions required to achieve the objectives of the trial is dictated by statistical power analysis. The

15 number of patients required for a given pharmacogenetic clinical trial will be determined on the prior knowledge of but not exclusively limited to variance or haplotype frequency, actual disease, disorder, or condition causing allele or allele associated with the disease, disorder, or condition and their linkage relationships. For a large scale pharmacogenetic clinical study, the identified sample size will require an adequate analysis of the

20 frequency of the allelic variance or variances within a given population, as described, for example, by Tu & Whitkemore (1999) and references therein.

Clinical trials can be designed to obscure the human subjects and/or the study coordinators from biasing that may occur during the testing of a candidate therapeutic invention. Often the candidate therapeutic intervention is compared to best medical

25 treatment, or a placebo (a compound, agent, device, or procedure that appears identical to the candidate therapeutic intervention but is innocuous to the receiving subject). Thus, control with placebo limits efficacy perception by influencing factors such as prejudice on the part of the study participant or investigator, spontaneous alterations or variations that occur during treatment and are related to the disease studied, or are unrelated to the

30 candidate therapeutic intervention. In pharmacogenetic clinical studies, a placebo arm or best medical therapy may be required in order to ascertain the effect of the allelic variance or variances on the efficacy or toxicology of the candidate therapeutic intervention.

Blinding refers to the lack of knowledge of the identity of the trial treatment and

35 thus can be used to ascertain the real and not perceived effects of the candidate therapeutic intervention. Patients, trial subjects, investigators, data review committees, ancillary personnel, statisticians, and clinical trial monitors may be blinded or unblinded during the trial period. Open label trials refer to those that are unblinded; single blind is

when the patient is kept unaware of the treatment groups; double blind is when both the patient and the investigator is kept unaware of the treatment groups; or a combination of these may be instituted during the trial period. Pharmacogenetic clinical trial design may include one or a combination of open label, single blind, or double blind clinical trial

5 design because reduction of inherent biases due to the knowledge of the type of treatment the human subject or the patient is to receive will ensure detection of the accuracy of the benefits of the stratification based upon allelic variance or variances or haplotypes.

In the designed studies in all four phases, termination endpoints for trials

10 including or excluding pharmacogenetic objectives are defined and include observation of adverse clinical events, voluntary lack of study participation either in the form of lack of adherence to the clinical protocol or sudden change in lifestyle of the participant, lack of adherence on the part of trial investigators to follow the trial protocol, death, or lack of efficacy or positive response within the test group.

15 Phase I of clinical development is a safety study performed in a limited (< 15) number of normal, healthy volunteers usually at single institutions. The primary endpoints in these studies is to determine pharmacokinetic parameters (i.e. adsorption, distribution, and bioavailability), dose-related side effects that are either desirable or undesirable, and metabolites that corroborate preclinical animal studies. In a Phase I

20 pharmacogenetic clinical trial, stratification based upon allelic variance or variances of a suspected gene or genes involving any or all of the pharmacokinetic parameters will be considered and incorporated in the objectives of the trial design.

In some cases, a pharmacogenetic Phase I study may enroll healthy human volunteers and stratify these individuals based upon their genotype. In this case, a study

25 objective may include observation of the effect of the allele/haplotype (detectable or undetectable) which the candidate therapeutic intervention may exhibit within the allelic variance, allelic variances, or haplotype groupings which can be assessed in the absence of a disease, disorder, or condition.

In some cases (e.g. cancer or medically intractable, life threatening, for those in

30 which no medical alternative exists, or seriously debilitating diseases, disorders, or conditions) Phase I studies can include a limited number of patients with a diagnosed disease, disorder, or condition for whom clinical parameters satisfy a specified inclusion criteria (see below). These safety/limited efficacy studies can be conducted at multiple institutions to ensure enrollment of these patients. In a pharmacogenetic Phase I study

35 that will include patients to some degree, the gene or genes and allelic variance or variances suspected to be involved in the efficacy of the candidate therapeutic intervention will be considered in the design of the inclusion criteria, the objectives, and the primary endpoints.

Phase II studies include a limited number of patients (<100) that satisfy the required inclusion criteria and do not satisfy any of the exclusion criteria of the trial design. Phase II studies can be conducted at single or multiple institutions. Inclusion criteria for patient enrollment to a clinical trial is a list of qualities for a given patient

5   population that includes pathophysiologic clinical parameters for a given disease, disorder, or condition that can be determined by clinical diagnosis or laboratory or diagnostic test; age; gender; fertility state (e.g. pre- or postmenopausal women); coexisting medical therapies; or psychological, emotional, or cognitive state. Inclusion criteria can also include defined psychological, emotional, or socioeconomic support by

10   family or friends. Exclusion criteria for patient enrollment generally includes the listing of co-morbidities that may interfere with the observations of the medical or laboratory pathophysiological clinical parameters of the disease, disorder, or condition, age, gender, fertility state (e.g. pre- or postmenopausal women), or previous or concurrent medical, surgical, or diagnostic therapies. In Phase II, the primary endpoint of the study is

15   generally limited efficacy and corroboration of the Phase I safety data in the specified patient population defined by the inclusion/exclusion criteria of the clinical protocol. Primary efficacy endpoints include observed improvements of pathophysiologic parameters that are determined medically, diagnostically (e.g. clinical laboratory values), or by surrogate measurements of the pathological state of the disease, disorder, or

20   condition. Primary endpoints may also include limitation of pharmacologic therapies, reduction of time to death, or reduction in the progression of the disease, disorder, or condition. Surrogate markers are pathophysiologic parameters determined by medical or clinical laboratory diagnosis that are associated and have been correlated with the prognosis, progression, predisposition, or risk analysis with a disease, disorder, or

25   condition that are not directly related to the primary diagnosed pathophysiologic condition, e.g. lowering blood pressure and coronary heart disease. Secondary endpoints are those that supplement the primary endpoint and can be used to support further clinical studies. For example, secondary endpoints include reduction in pharmacologic therapy, reduction in requirement of a medical device, or alteration of the progression of the

30   disease disorder, or condition. Typically, in Phase II, treatment groups with varying doses are included in the study to identify the appropriate dosage and pharmacokinetic parameters to achieve maximum efficacy.

In a pharmacogenetic Phase II clinical trial, retrospective or prospective design will include the stratification of the patients based upon suspected gene or genes and

35   allelic variance or variances involved in the pathway for pharmacodynamic or pharmacokinetic response demonstrated in the treatment groups of the candidate therapeutic intervention. These pharmacodynamic parameters may include surrogate endpoints, efficacy endpoints, or pathophysiologic thresholds. Pharmacokinetic

parameters may include but are not exclusive of dosage, toxicological variables, metabolism, or excretion. Other parameters that may effect the outcome of a pharmacogenetic clinical trial may include gender, race, ethnic origins (population history), and combination of allelic variances of genes from multiple pathways, leading to

5   but not exclusively efficacy or toxicology.

Phase III studies include multi-site, large, statistically significant, numbers of patients (<5,000) that fulfill the inclusion criteria for the study. The design of this type of trial includes power analysis to ensure the data will support the study objectives. In this large scale efficacy study, the primary endpoint is preferably defined as enhanced

10   efficacy as compared to placebo or best medical care for said disease, disorder, or condition. The primary endpoint may include reduction of condition progression, improvement of a specific subset of symptoms, or in requirement or perceived need of medical therapy. In a pharmacogenetic Phase III clinical study, the endpoints will be the determination of the efficacy or toxicological differences that can be demonstrated to be

15   dependent on the stratification based upon allelic variance or variances in a gene or genes that are suspected to be involved in the efficacy or toxicological population phenotype. Further in the Phase III pharmacogenetic clinical trial, the analysis of the impact of the allelic variance or variances will be broadened from the confirmatory Phase II pharmacogenetic clinical trial data that supports the notion that the phenotypic response

20   differences can be identified as dependent on the allelic variance or variances of a gene or genes suspected to be involved in the efficacy or toxicological response.

After the completion of a Phase III study, the data and information from all of the trials are compiled into a New Drug Application for review by the US FDA for marketing approval in the US and its territories. The NDA includes the raw (unanalyzed) clinical

25   data, i.e. the primary endpoints or secondary endpoints, a statistical analysis of all of the included data, a document describing in detail any adverse or observed side effects, tabulation of the participant drop-outs and detailed reasons for the termination, and other specific data or details of ongoing *in vitro* or *in vivo* studies since the submission of the IND. If pharmacoeconomic objectives are a part of the clinical trial design data

30   supporting cost or economic analyses are included in the NDA. In a pharmacogenetic clinical study, the pharmacoeconomic analyses may include demonstration or lack of benefit of the candidate therapeutic intervention in a cost benefit analysis, cost of illness study, cost minimization study, or cost utility analysis. In one or a combination of these studies, the effect of a diagnostic identification of the population and subsequent

35   stratification based upon allelic variance or variances or haplotype of a suspected gene or genes involved in the efficacy or toxicological responses of the candidate therapeutic intervention will be used to support application for the approval for the marketing and sale of the candidate therapeutic intervention.

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Phase IV studies occur after the therapeutic intervention has been approved for marketing. In these studies, retrospective data and data from a large patient population that do not necessarily fulfill the pathophysiologic requirements of the approved indication are included. In a Phase IV pharmacogenetic clinical trial, both retrospective

5 and prospective design can be incorporated. In both cases, stratification based upon allelic variance or variances with adequate sample size in order to determine the statistical relevance of an outcome difference among the treatment groups.

Although the above listed phases of clinical development are well-established, there are cases whereby strict Phase I, II, III development does not occur, i.e. the clinical

10 development of candidate therapeutic interventions for serious debilitating or life threatening diseases, or for those cases whereby no medical therapeutic alternative exists. In the cases whereby the target indication for cancer or medically intractable, life threatening or seriously debilitating diseases, disorders, or conditions the US FDA has regulatory procedural mechanisms that can expedite the availability of the therapeutic

15 intervention for patients that fall into one or more of these categories. Such development incentives include Treatment IND, Fast-Track or Accelerated review, and Orphan Drug Status. In a pharmacogenetic clinical development program for candidate therapeutic interventions for this class of indications, consideration of sample size for adequate determination of the effect allelic variance or variances may have on the outcome

20 response or endpoints is incorporated. Further consideration may include but is not limited to accrual rate for candidate patients, and number of institutions or clinical sites required to achieve an appropriate sample size.

In additional cases of diseases, disorders, or conditions where there are no therapeutic alternatives development, sponsors may choose to expedite the development

25 of the candidate therapeutic intervention without making use of the above FDA regulatory clinical development incentives. In these cases, the sponsor proposes expedited clinical development of a candidate therapeutic intervention due to outstanding positive or unequivocal preclinical safety and/or efficacy data.

As used herein, "supplemental applications" are those in which a candidate

30 therapeutic intervention is tested in a human clinical trial in order for the product to have an expanded label to include additional indications for therapeutic use. In these cases, the previous clinical studies of the therapeutic intervention, i.e. those involving the preclinical safety and Phase I human safety studies can be used to support the testing of the particular candidate therapeutic intervention in a patient population for a different

35 disease, disorder, or condition than that previously approved in the US. In these cases, a limited Phase II study is performed in the proposed patient population. With adequate signs of efficacy, a Phase III study is designed. All other parameters of clinical development for this category of candidate therapeutic interventions proceeds as

described above for interventions first tested in human candidates.

    As used herein, "outcomes" or "therapeutic outcomes" are used to describe the results and value of healthcare intervention. Outcomes can be multi-dimensional, e.g., including one or more of the following: improvement of symptoms; regression of the disease, disorder, or condition; economic outcomes of healthcare decisions.

    As used herein, "pharmacoeconomics" is the analysis of a therapeutic intervention in a population of patients diagnosed with a disease, disorder, or condition that includes at least one of the following studies: cost of illness study (COI); cost benefit analysis (CBA), cost minimization analysis (CMA), or cost utility analysis (CUA), or an analysis comparing the relative costs of a therapeutic intervention with one or a group of other therapeutic interventions. In each of these studies, the cost of the treatment of a disease, disorder, or condition is compared among treatment groups. As used herein, costs are those economic variables associated with a disease, disorder, or condition fall into two broad categories: direct and indirect. Direct costs are associated with the medical and non-medical resources used as therapeutic interventions, including medical, surgical, diagnostic, pharmacologic, devices, rehabilitation, home care, nursing home care, institutional care, and prosthesis. Indirect costs are associated with loss of productivity due to the disease, disorder, or condition suffered by the patient or relatives. A third category, the tangible and intangible losses due to pain and suffering of a patient or relatives often is included in indirect cost studies.

    As used herein, "health-related quality of life" is a measure of the impact of the disease, disorder, or condition on an individual's or group of patient's activities of daily living. Preferably, included in pharmacoeconomic studies is an analysis of the health-related quality of life. Standardized surveys or questionnaires for general health-related quality of life or disease, disorder, or condition specific determine the impact the disease, disorder, or condition has on an individuals day to day life activities or specific activities that are affected by a particular disease, disorder, or condition.

    As used herein, the term "stratification" refers to the creation of a distinction between patients on the basis of a characteristic or characteristics of the patient. Generally, in the context of clinical trials, the distinction is used to distinguish responses or effects in different sets of patients distinguished according to the stratification parameters. For the present invention, stratification preferably includes distinction of patient groups based on the presence or absence of particular variance or variances in one or more genes. The stratification may be performed only in the course of analysis or may be used in creation of distinct groups or in other ways.

    A human clinical trial can result in data to support the utility of a gene variance or variances for the selection of optimal therapy. Clinical studies require no knowledge of the biological function of the gene containing the variance of the variances to be assessed,

nor any knowledge of how the therapeutic invention to be assessed works at a biochemical level.

There are several important preclinical data sets that pose criteria to consider when designing a clinical study to assess the utility of a variance in a gene for selecting optimal therapy for a disease, disorder, or condition. Preferably, the data sets include one or a combination of at least of the following:

Mechanism of action of the therapeutic intervention-
If the candidate therapy (e.g. drug) has established mechanism of action, the target genes can be appropriately identified. *In vitro* data supporting altered physiologic activity of the variant forms of the gene in the presence of the therapy, assists the direction of the fundamental hypotheses and identifying the objectives for a human clinical trial.

Mechanism of metabolic transformation of the therapeutic intervention-
If *in vitro* or *in vivo* animal studies have demonstrated metabolic biotransformation of the therapeutic intervention, correlation of the effects of a variance or variances on the metabolic biotransformation of the therapeutic intervention can further assist the direction of the fundamental hypotheses and identification of the objectives of the human clinical study.

Effect of the variance or variances on therapeutic intervention-
The combined preclinical data sets should point to the premise of a controlled clinical trial of the the therapeutic intervention. The design of the trial will preferably incorporate the preclinical data sets to determine the primary and secondary endpoints. Preferably, these endpoints will include whether the therapeutic intervention is efficacious, efficacious with undesirable side effects, ineffective, ineffective with undesirable side effects, or ineffective with deleterious effects. Pharmacoeconomic analyses may be incorporated in order to support the efficacious intervention, efficacious with undesirable side effects cases, whereby the clinical outcome is positive, and economic analyses are required for the support of overall benefit to the patient and to society.

The strategies for designing a clinical trial to test the effect of a genotypic variance or variances on a physiological response to therapeutic intervention for drugs with known mechanism of action, mechanism of biotransformation, and/or known physiologic response differentials correlated to genotypic variance or variances will be modified based upon the data and information from the preclinical studies and the patient symptomatic parameters unique to the target indication. However, the strategy (design) and the implementation (conduct) of the clinical study preferably consist of one or more of the following strategies.

A. Retrospective clinical trials.

In general the goal of retrospective clinical trials will be to test and refine hypotheses regarding genetic factors that are associated with drug responses. The best supported hypotheses can subsequently be tested in prospective clinical trials, and data from the prospective trials will likely comprise the main basis for an application to register the drug and predictive genetic test with the appropriate regulatory body. In some cases, however, it may become acceptable to use data from retrospective trials to support regulatory filings.

I.   Clinical trials to study the effect of one gene locus on drug response

A. Stratify patients by genotype at one candidate variance in the candidate gene locus.

1. Genetic stratification of patients can be accomplished in several ways, including the following (where 'A' is the more frequent form of the variance being assessed and 'a' is the less frequent form):

(a) AA vs. aa

(b) AA vs. Aa vs. aa

(c) AA vs. (Aa + aa)

(d) (AA + Aa) vs. aa.

2.   The effect of genotype on drug response phenotype may be affected by a variety of nongenetic factors. Therefore it may be beneficial to measure the effect of genetic stratification in a subgroup of the overall clinical trial population. Subgroups can be defined in a number of ways including, for example, biological, clinical, pathological or environmental criteria. For example, the predictive value of genetic stratification can be assessed in a subgroup or subgroups defined by:

a.   Biological criteria:

i.   gender (males vs. females)

ii.   age (for example above 60 years of age). Two, three or more age groups may be useful for defining subgroups for the genetic analysis.

iii.   hormonal status and reproductive history, including pre- vs. post- menopausal status of women, or multiparous vs. nulliparous women

iv.   ethnic, racial or geographic origin, or surrogate markers of ethnic, racial or geographic origin. (For a description of genetic markers that serve as surrogates of racial/thnic origin see, for example: Rannala, B. and J.L. Mountain, Detecting immigration by using multilocus genotypes. *Proc*

Natl Acad Sci U S A , 94 (17): 9197-9201, 1997. Other surrogate markers could be used, including biochemical markers.)

       b.   Clinical criteria:

       i.   Disease status.   There are clinical grading scales for many diseases. For example, the status of Alzheimer's Disease patients is often measured by cognitive assessment scales such as the mini-mental status exam (MMSE) or the Alzheimer's Disease Assessment Scale (ADAS), which includes a cognitive component (ADAS-COG). There are also clinical assessment scales for many other diseases, including cancer.

       ii.   Disease manifestations (clinical presentation).

       c.   Pathological criteria:

       i.   Histopathologic features of disease tissue, or pathological diagnosis. (For example there are many varieties of lung cancer: squamous cell carcinoma, adenocarcinoma, small cell carcinoma, bronchoalveolar carcinoma, etc., each of which may – which, in combination with genetic variation, may correlate with

       ii.   Pathological stage. A variety of diseases have pathological staging schemes

       iii.   Loss of heterozygosity (LOH)

       iv.   Pathology studies such as measuring levels of a marker protein

       v.   Laboratory studies such as hormone levels, protein levels, small molecule levels

       3.   Measure frequency of responders in each genetic subgroup. Subgroups may be defined in several ways.

       i.   more than two age groups

       ii.   age related status such as pre or post-menopausal

       Stratify by haplotype at one candidate locus where the haplotype is made up of two variances, three variances or greater than three variances.

4.   ***Statistical analysis of clinical trial data***

       There are a variety of statistical methods for measuring the difference between two or more groups in a clinical trial. One skilled in the art will recognize that different methods are suited to different data sets. In general, there is a family of methods customarily used in clinical trials, and another family of methods customarily used in genetic epidemiological studies. Methods from either family

may be suitable for performing statistical analysis of pharmacogenetic clinical trial data.

a. Conventional Clinical Trial Statistics

5   Conventional clinical trial statistics include hypothesis testing and descriptive methods, as elaborated below.   Guidance in the selection of appropriate statistical tests for a particular data set can be obtained from texts such as: Biostatistics: A Foundation for Analysis in the Health Sciences, 7th edition (Wiley Series in Probability and Mathematical Statistics, Applied Probability and statistics)

10   by Wayne W. Daniel, John Wiley & Sons, 1998; Bayesian Methods and Ethics in a Clinical Trial Design (Wiley Series in Probability and Mathematical Statistics. Applied Probability Section) by J. B. Kadane (Editor), John Wiley & Sons, 1996;

b. Hypothesis testing statistical procedures

15   (1)   One-sample procedures (binomial confidence interval, Wilcoxon signed rank test, permutation test with general scores, generation of exact permutational distributions)

(2)   Two-sample procedures (t-test, Wilcoxon-Mann-Whitney test,
20   Normal score test, Median test, Van der Waerden test, Savage test, Logrank test for censored survival data, Wilcoxon-Gehan test for censored survival data, Cochran-Armitage trend test, permutation test with general scores, generation of exact permutational distributions)

25   (3)   R x C contingency tables (Fisher's exact test, Pearson's chi-squared test, Likelihood ratio test, Kruskal-Wallis test, Jonckheere-Terpstra test, Linear-by linear association test, McNemar's test, marginal homogeneity test for matched pairs)

30   (4)   Stratified 2 x 2 contingency tables (test of homogeneity for odds ratio, test of unity for the common odds ratio, confidence interval for the common odds ratio)

(5)   Stratified 2 x C contingency tables (all two-sample procedures listed
35   above with stratification, confidence intervals for the odds ratios and trend, generation of exact permutational distributions)

(6) General linear models (simple regression, multiple regression, analysis of variance –ANOVA-, analysis of covariance, response-surface models, weighted regression, polynomial regression, partial correlation, multiple analysis of variance -MANOVA-, repeated measures analysis of variance).

5

(7) Analysis of variance and covariance with a nested (hierarchical) structure.

(8) Designs and randomized plans for nested and crossed experiments
10 (completely randomized design for two treatment, split-splot design, hierarchical design, incomplete block design, latin square design)

(9) Nonlinear regression models

15 (10) Logistic regression for unstratified or stratified data, for binary or ordinal response data, using the logit link function, the normit function or the complementary log-log function.

(11) Probit, logit, ordinal logistic and gompit regression models.
20

(12) Fitting parametric models to failure time data that may be right-, left-, or interval-censored. Tested distributions can include extreme value, normal and logistic distributions, and, by using a log transformation, exponential, Weibull, lognormal, loglogistic and gamma distributions.

25

(13) Compute non-parametric estimates of survival distribution with right-censored data and compute rank tests for association of the response variable with other variables.

30   c. Descriptive statistical methods
   • Factor analysis with rotations
   • Canonical correlation
   • Principal component analysis for quantitative variables.
   • Principal component analysis for qualitative data.
35   • Hierarchical and dynamic clustering methods to create tree structure, dendrogram or phenogram.
       • Simple and multiple correspondence analysis using a contingency table as input or raw categorical data.

Specific instructions and computer programs for performing the above
calculations can be obtained from companies such as: SAS/STAT Software, SAS
Institute Inc., Cary, NC, USA; BMDP Statistical Software, BMDP Statistical
5   Software Inc., Los Angeles, CA, USA; SYSTAT software, SPSS Inc., Chicago, IL,
USA; StatXact & LogXact, CYTEL Software Corporation, Cambridge, MA, USA.

d. Statistical Methods from Genetic Epidemiology

Genetic epidemiological methods can also be useful in carrying out statistical
10   tests for the present invention.

Guidance in the selection of appropriate genetic statistical tests for analysis
of a particular data set can be obtained from texts such as: Fundamentals of Genetic
Epidemiology (Monographs in Epidemiology and Biostatistics, Vol 22) by M. J.
Khoury, B. H. Cohen & T. H. Beaty, Oxford Univ Press, 1993; Methods in Genetic
15   Epidemiology by Newton E. Morton, S. Karger Publishing, 1983; Methods in
Observational Epidemiology, 2nd edition (Monographs in Epidemiology and
Biostatistics, V. 26) by J. L. Kelsey (Editor), A. S. Whittemore & A. S. Evans, 1996;
Clinical Trials : Design, Conduct, and Analysis (Monographs in Epidemiology and
Biostatistics, Vol 8) by C. L. Meinert & S. Tonascia, 1986)
20

**Strategy for the implementation of a clinical study in the case of a
therapeutic with known mechanism of action:**

1.   Identify genes that encode proteins that perform functions related to drug
25   absorption and/or, distribution, as well as genes related to the pharmacological
action (pharmacodynamics) of the therapeutic intervention. Genes that encode
proteins homologous to the proteins believed to carry out the above functions are
also worth evaluation as they may carry out similar functions. Together the
foregoing proteins constitute the candidate genes for affecting response of a patient
30   to the therapeutic intervention.

2.   Identify variances in the candidate genes. Initially, individual variances (and
preferably their frequencies) will be identified by standard methods. Then, for genes
with more than one variance, the commonly occurring patterns of variances
occurring on a single chromosome (i.e. the haplotypes) may also be established
35   using both computational and experimental approaches. For example, a
computational approach might include one of, but not limited to, the following two
methods a) expectation maximization (E-M) algorithm (Excoffier and Slatkin, Mol.
Biol. Evol. 1995) and, b) a combination of Parsimonious and E-M methods.

If we have a large population, implementation of the E-M method will be performed first.

A given phenotype or a sequence could come from several genotypes. This is particularly true if the sequence is heterozygous at a number of nucleotide positions. Therefore, it is not practical to just count the phenotypes and make a conclusion on the underlying genotype, because it may lead to ambiguities. To avoid such ambiguities, an alternative iterative method called the EM (expectation-maximization) algorithm is used to derive the expected genotypes for a given phenotype or a sequence. This method assumes that the population under consideration is in Hardy-Weinberg equilibrium.

For example, consider the *ABO* locus in a population. Supposing, there are *Na* people of type *A*, *Nb* people of type *B*, *Nab* people of type *AB*, and *No* people of type *O*. Assuming $N = Na + Nb + Nab + No$ in the random sample of people *N*, we cannot tell exactly how many of the *Na* people are homozygous for *A/A* and how many are heterozygotes for *A/O*.

In order to avoid this dilemma, we first assume that the expected number of genotypic frequencies in the population is in H-W equilibrium for any given (all) allele(s) frequency. This is followed by setting the allele frequencies and iteration *n*, and testing for its stability in a series of iterations, up to *m*. When the values of the initial allele frequencies stabilize at the end of series of iterations up to *m*, the resulting expected number of genotypes are assigned to phenotypes; for example, sequences or individuals.

The following steps are involved in the E-M algorithm:

1. Chose an allele or a haplotype in an expected class that occurs at the highest frequency

2. Use it as a base for the observed values and estimate the unobserved or the expected value

3. Use the second value as the true value and estimate the unobserved value from the second value

4. Continue this process (up to *m*) till you find values that do not change from one iteration to the next.

5. The final value is the maximum likelihood (highly likely) estimate of that allele or the haplotype

As indicated above, also among the number of methods which are used for the purpose of classifying DNA sequences, haplotypes or phenotypic characters are the parsimony methods. Parsimony principle maintains that the best explanation for

the observed differences among sequences, phenotypes (individuals, species) etc., is provided by the smallest number of evolutionary changes. Alternatively, simpler hypotheses are preferable to explain a set of data or patterns, than more complicated ones, and that *ad hoc* hypotheses should be avoided whenever possible (Molecular

5 Systematics, Hillis et al., 1996). These methods for inferring relationship among sequences operate by minimizing the number of evolutionary steps or mutations (changes from one sequence/character) required to explain a given set of data.

For example, supposing we want to obtain relationships among a set of sequences and construct a structure (tree/topology), we first count the minimum

10 number of mutations that are required for explaining the observed evolutionary changes among a set of sequences. A structure (topology) is constructed based on this number. When once this number is obtained, another structure is tried. This process is continued for all reasonable number of structures. Finally, the structure that required the smallest number of mutational steps is chosen as the likely

15 structure/evolutionary tree for the sequences studied.

If the computed frequency of the haplotypes are equal to the number of individuals in the population, then there will be a consideration of utilizing additional methods. For these cases and if there is a small population, then the

20 number of haplotypes will be considered relative to the number of entrants. In a method that is a modification of previously published work (Clark, Mol Biol and Evol. 1990) homozygotes will be assigned one unambiguous haplotype. If there is a single site variance (mutation) at one of the chromosomes then it will have two haplotypes. As the number of variances (mutations) increase in the diploid

25 chromosomes, each of these variances will be compared with the haplotypes of the original population. Then a frequency will be assigned to the new variance based upon the Hardy-Weinberg expected frequencies. (See text below for why haplotypes are useful and how to determine them experimentally, if necessary.)

3. Retrospectively reanalyze data from already completed clinical trials. Since

30 the questions are new, the data can be treated as if it were a prospective trial, with identified variances or haplotypes as stratification criteria and biological/clinical endpoints. Care should be taken to avoid studying a population in which there may be a link between drug-related genes and disease-related genes.

4. Select group of variances or haplotypes to differentiate: one control group

35 including groups of variances with normal biological response one or a few case groups including groups of variances with significant biological impact

5. Establish phase III trials with selected variances as inclusion criteria and clinical/pharmacoeconomic endpoints. The number of patients required for adequate

statistical power (approximately the same as in a usual phase III trial) will be determined from the phase II results and allele frequencies.

Strategy for the implementation of a clinical study in the case of a therapeutic intervention with known mechanism of biotransformation:

1.   Identify genes that encode proteins that perform functions related to drug biotransformation or excretion, as well as genes related to the pharmacological action (pharmacodynamics) of the metabolized or biotransformed therapeutic intervention. Genes that encode proteins homologous to the proteins believed to carry out the above functions are also worth evaluation as they may carry out similar functions. Together the foregoing proteins constitute candidate genes for affecting response of a patient to the therapeutic intervention.

2.   Identify variances in the candidate genes. Initially, individual variances will be identified by standard methods. Then, for genes with more than one variance, the commonly occurring patterns of variances occurring on a single chromosome (i.e. the haplotypes) may also be established. (See text below for why haplotypes are useful and how to determine them experimentally, if necessary.)

3.   Retrospectively reanalyze data from already completed clinical trials. Since the questions are new, the data can be treated as if it were a prospective trial, with identified variances or haplotypes as stratification criteria and biological/clinical endpoints. Care should be taken to avoid studying a population in which there may be a link between drug-related genes and disease-related genes.

4.   Select group of variances or haplotypes to differentiate: one control group including groups of variances with normal biological response one or a few case groups including groups of variances with significant biological impact.

5.   Establish phase III trials with selected variances as inclusion criteria and clinical/pharmacoeconomic endpoints. The number of patients required for adequate statistical power (approximately the same as in a usual phase III trial) will be determined from the phase II results and allele frequencies.

Strategy for the implementation of a clinical study in the case of a therapeutic intervention where by the effect of the gene variance or variances on therapeutic intervention is known:

1.   Retrospectively reanalyze data from already completed clinical trials. In this case, since the questions are new, the data can be treated as if it were a prospective trial, with identified variances or haplotypes as stratification criteria and biological/clinical endpoints. Care should be taken to avoid studying a population in

which there may be a link between drug-related genes and disease-related genes.

2.   Select group of variances or haplotypes to differentiate: one control group including groups of variances with normal biological response and one or a few case groups including groups of variances with significant biological impact.

5   3.   Establish phase III or phase IV (post marketing) trials with selected variances as inclusion criteria and clinical/pharmacoeconomic endpoints. The number of patients required for adequate statistical power (approximately the same as in a usual phase III trial) will be determined from the phase II results and allele frequencies.

10   A clinical trial in which pharmacogenetic related efficacy or toxicity endpoints are included in the primary or secondary endpoints will be part of a retrospective or prospective clinical trial. In the design of these trials, the allelic differences will be identified and stratification based upon these genotypic differences among patient or subject groups will be used to ascertain the significance

15   of the impact a genotype has on the candidate therapeutic intervention. Retrospective pharmacogenetic trials can be conducted at each of the phases of clinical development, with the assumption that sufficient data is available for the correlation of the physiologic effect of the candidate therapeutic intervention and the allelic variance or variances within the treatment population. In the case of a

20   retrospective trial, the data collected from the trial can be re-analyzed by imposing the additional stratification on groups of patients by specific allelic variances that may exist in the treatment groups. Retrospective trials can be useful to ascertain whether a hypothesis that a specific variance has a significant effect on the efficacy or toxicity profile for a candidate therapeutic intervention.

25   A prospective clinical trial has the advantage that the trial can be designed to ensure the trial objectives can be met with statistical certainty. In these cases, power analysis, which includes the parameters of allelic variance frequency, number of treatment groups, and ability to detect positive outcomes can ensure that the trial objectives are met.

30   In designing a pharmacogenetic trial, retrospective analysis of Phase II or Phase III clinical data can indicate trial variables for which further analysis is required. For example, surrogate endpoints, pharmacokinetic parameters, dosage, efficacy endpoints, ethnic and gender differences, and toxicological parameters may result in data that would require further analysis and re-examination through the

35   design of an additional trial. In these cases, analysis involving statistics,, genetics, clinical outcomes, and economic parameters may be considered prior to proceeding to the stage of designing any additional trials. Factors involved in the consideration of statistical significance may include Bonferroni analysis, permutation testing, with

multiple testing correction resulting in a difference among the treatment groups that has occurred as a result of a chance of no greater than 20%, i.e. p< 0.20. Factors included in determining clinical outcomes to be relevant for additional testing may include, for example, consideration of the target indication, the trial endpoints,

5   progression of the disease, disorder, or condition during the trial study period, biochemical or pathophysiologic relevance of the candidate therapeutic intervention, and other variables that were not included or anticipated in the initial study design or clinical protocol. Factors to be included in the economic significance in determining additional testing parameters include sample size, accrual rate, number of clinical

10   sites or institutions required, additional or other available medical or therapeutic interventions approved for human use, and additional or other available medical or therapeutic interventions concurrently or anticipated to enter human clinical testing. Further, there may be patients within the treatment categories that present data that fall outside of the average or mean values, or there may be an indication of multiple

15   allelic loci that are involved in the responses to the candidate therapeutic intervention. In these cases, one could propose a prospective clinical trial having an objective to determine the significance of the variable or parameter and its effect on the outcome of the parent Phase II trial. In the case of a pharmacogenetic difference, i.e. a single or multiple allelic difference, a population could be selected based upon

20   the distribution of genotypes. The candidate therapeutic intervention could then be tested in this group of volunteers to test for efficacy or toxicity. The repeat prospective study could be a Phase I limited study in which the subjects would be healthy human volunteers, or a Phase II limited efficacy study in which patients which satisfy the inclusion criteria could be enrolled. In either case, the second,

25   confirmatory trial could then be used to systematically ensure an adequate number of patients with appropriate phenotype is enrolled in a Phase III trial.

A placebo controlled pharmacogenetics clinical trial design will be one in which target allelic variance or variances will be identified and a diagnostic test will be performed to stratify the patients based upon presence, absence, or combination

30   thereof of these variances. In the Phase II or Phase III stage of clinical development, determination of a specific sample size of a prospective trial will be described to include factors such as expected differences between a placebo and treatment on the primary or secondary endpoints and a consideration of the allelic frequencies.

The design of a pharmacogenetics clinical trial will include a description of

35   the allelic variance impact on the observed efficacy between the treatment groups. Using this type of design, the type of genetic and phenotypic relationship display of the efficacy response to a candidate therapeutic intervention will be analyzed. For example, a genotypically dominant allelic variance or variances will be those in

which both heterozygotes and homozygotes will demonstrate a specific phenotypic efficacy response different from the homozygous recessive genotypic group. A pharmacogenetic approach is useful for clinicians and public health professionals to include or eliminate small groups of responders or non-responders from treatment in order to avoid unjustified side-effects. Further, adjustment of dosages when clear clinical difference between heterozygous and homozygous individuals may be beneficial for therapy with the candidate therapeutic intervention

In another example, a reccesive allelic variance or variances will be those in which only the homozygote recessive for that or those variances will demonstrate a specific phenotypic efficacy response different from the heterozygotes or homozygous dominants. An extension of these examples may include allelic variance or variances organized by haplotypes from additional gene or genes providing an explanation of clinical phenotypic outcome differences among the treatment groups. These types of clinical studies will point and address allelic variance and its role in the efficacy or toxicology pattern within the treatment population.

IV. Variance Identification and Use

A. Initial Identification of variances in genes

Selection of population size and composition

Prior to testing to identify the presence of sequence variances in a particular gene or genes, it is useful to understand how many individuals should be screened to provide confidence that most or nearly all pharmacogenetically relevant variances will be found. The answer depends on the frequencies of the phenotypes of interest and what assumptions we make about heterogeneity and magnitude of genetic effects. At the beginning we only know phenotype frequencies (e.g. responders vs. nonresponders, frequency of various side effects, etc.). As an example, the occurrence of serious 5-FU/FA toxicity - e.g. toxicity requiring hospitalization is often >10%. The occurrence of life threatening toxicity is in the 1-3% range (Buroker et al. 1994). The occurrence of complete remissions is on the order of 2-8%. The lowest frequency phenotypes are thus on the order of ~2%. If we assume that (i) homogeneous genetic effects are responsible for half the phenotypes of interest and (ii) for the most part the extreme phenotypes represent recessive genotypes, then we need to detect alleles that will be present at ~10% frequency (.1 x .1 = .01, or 1% frequency of homozygotes) if the population is at Hardy-Weinberg equilibrium. To have a ~99% chance of identifying such alleles would require searching a

population of 22 individuals (see Table 1 below). If the major phenotypes
are associated with heterozygous genotypes then we need to detect alleles
present at ~.5% frequency (2 x .005 x .995 = .00995, or ~1% frequency of
heterozygotes). A 99% chance of detecting such alleles would require ~40
individuals (Table below). Given the heterogeneity of the North American
population we cannot assume that all genotypes are present in Hardy-
Weinberg proportions, therefore a substantial oversampling is done to
increase the chances of detecting relevant variances: For our initial screening,
usually, 62 individuals of known race/ethnicity are screened for variance.
Variance detection studies can be extended to outliers for the phenotypes of
interest to cover the possibility that important variances were missed in the
normal population screening.

Table 1

Allele frequencies	Number of subjects genotyped							
	n = 5	n = 10	n = 15	n = 20	n = 25	n = 30	n = 35	n = 50
p=.99,	9.56 %	18.21	26.03	33.10	39.50	45.28	50.52	63.40
p=.97,	26.26	45.62	59.90	70.43	78.19	83.92	88.14	95.24
p=.95,	40.13	64.15	78.53	87.15	92.30	95.39	97.24	99.65
p=.93,	51.60	76.58	88.66	94.51	97.34	98.71	99.38	99.93
p =.9, q =	65.13	87.84	95.76	98.52	99.48	99.82	99.94	>99.9
p =.8, q =	89.26	98.84	99.88	99.99	>99.9	>99.99	>99.9	>99.9
p =.7, q =	97.17	99.92	99.99	>99.99	>99.9	>99.99	>99.9	>99.9

*Likelihood of Detecting Polymorphism in a Population as a Function
of Allele Frequency & Number of Individuals Genotyped*

        The table above shows the probability (expressed as percent) of
detecting both alleles (i.e. detecting heterozygotes) at a biallelic locus as a
function of (i) the allele frequencies and (ii) the number of individuals
genotyped. The chances of detecting heterozygotes increases as the
frequencies of the two alleles approach 0.5 (down a column), and as the
number of individuals genotyped increases (to the right along a row). The
numbers in the table are given by the formula: $1 - (p)^{2n} - (q)^{2n}$. Allele
frequencies are designated p and q and the number of individuals tested is
designated n. (Since humans are diploid, the number of alleles tested is
twice the number of individuals, or 2n.)

While it is preferable that numbers of individuals, or independent sequence samples, are screened to identify variances in a gene, it is also very beneficial to identify variances using smaller numbers of individuals or sequence samples. For example, even a comparison between the sequences

5   of two samples or individuals can reveal sequence variances between them. Preferably, 5, 10, or more samples or individuals are screened.

Source of nucleic acid samples

Nucleic acid samples, for example for use in variance identification,

10   can be obtained from a variety of sources as known to those skilled in the art, or can be obtained from genomic or cDNA sources by known methods. For example, the Coriell Cell Repository (Camden, N.J.) maintains over 6,000 human cell cultures, mostly fibroblast and lymphoblast cell lines comprising the NIGMS Human Genetic Mutant Cell Repository. A catalog

15   (http://locus.umdnj.edu/nigms) provides racial or ethnic identifiers for many of the cell lines. 55 of the 62 cell lines to be genotyped (as indicated above) are drawn from this collection; the remainder were obtained from the Beijing Cancer Institute. The cell lines are derived from 21 Caucasians (of Northern, Central and Southern European origin), 8 Afro-Americans, 9 Hispanics or

20   Mexicans, 8 Chinese, 12 Japanese, 1 American Indian, 1 East Indian, 1 Iranian, and 1 Korean. These cell lines (plus ~75 other lymphoblastoid lines) are currently in use by the inventors for variance detection studies.

Source of human DNA, RNA and cDNA samples

25   PCR based screening for DNA polymorphism can be carried out using either genomic DNA or cDNA produced from mRNA. For many genes, only cDNA sequences have been published, therefore the analysis of those genes is, at least initially, at the cDNA level since the determination of intron-exon boundaries and the isolation of flanking sequences is a laborious

30   process. However, screening genomic DNA has the advantage that variances can be identified in promoter, intron and flanking regions. Such variances may be biologically relevant. Therefore preferably, when variance analysis of patients with outlier responses is performed, analysis of selected loci at the genomic level is also performed. Such analysis would be contingent on the

35   availability of a genomic sequence or intron-exon boundary sequences, and would also depend on the anticipated biological importance of the gene in connection with the particular response.

When cDNA is to be analyzed it is very beneficial to establish a

tissue source in which the genes of interest are expressed at sufficient levels
that cDNA can be readily produced by RT-PCR. Preliminary PCR
optimization efforts for 19 of the 29 genes in Table 2 reveal that all 19 can be
amplified from lymphoblastoid cell mRNA. The 7 untested genes belong on
5   the same pathways and are expected to also be PCR amplifiable.

PCR Optimization

Primers for amplifying a particular sequence can be designed by
methods known to those skilled in the art, including by the use of computer
10   programs such as the PRIMER software available from Whitehead
Institute/MIT Genome Center. In some cases it is preferable to optimize the
amplification process according to parameters and methods known to those
skilled in the art; optimization of PCR reactions based on a limited array of
temperature, buffer and primer concentration conditions is utilized. New
15   primers are obtained if optimization fails with a particular primer set.

*Variance detection using T4 endonuclease VII mismatch cleavage
method*

Any of a variety of different methods for detecting variances in a
20   particular gene can be utilized, such as those described in the patents and
applications cited in section A above. An exemplary method is a T4
EndoVII method. The enzyme T4 endonuclease VII (T4E7) is derived from
the bacteriophage T4. T4E7 specifically cleaves heteroduplex DNA
containing single base mismatches, deletions or insertions. The site of
25   cleavage is 1 to 6 nucleotides 3' of the mismatch. This activity has been
exploited to develop a general method for detecting DNA sequence variances
(Youil et al. 1995; Mashal and Sklar, 1995). A quality controlled T4E7
variance detection procedure based on the T4E7 patent of R.G.H. Cotton and
co-workers. (Del Tito et al., in press) is preferably utilized. T4E7 has the
30   advantages of being rapid, inexpensive, sensitive and selective. Further,
since the enzyme pinpoints the site of sequence variation, sequencing effort
can be confined to a 25 -30 nucleotide segment.

The major steps in identifying sequence variations in candidate genes
using T4E7 are: (1) PCR amplify 400-600 bp segments from a panel of DNA
35   samples; (2) mix a fluorescently-labeled probe DNA with the sample DNA;
(3) heat and cool the samples to allow the formation of heteroduplexes; (4)
add T4E7 enzyme to the samples and incubate for 30 minutes at 37°C,
during which cleavage occurs at sequence variance mismatches; (5) run the

samples on an ABI 377 sequencing apparatus to identify cleavage bands, which indicate the presence and location of variances in the sequence; (6) a subset of PCR fragments showing cleavage are sequenced to identify the exact location and identity of each variance.

5   The T4E7 Variance Imaging procedure has been used to screen particular genes. The efficiency of the T4E7 enzyme to recognize and cleave at all mismatches has been tested and reported in the literature. One group reported detection of 81 of 81 known mutations (Youil et al. 1995) while another group reported detection of 16 of 17 known mutations (Mashal and

10   Sklar, 1995). Thus, the T4E7 method provides highly efficient variance detection.

DNA sequencing

          A subset of the samples containing each unique T4E7 cleavage site is

15   selected for sequencing. DNA sequencing can , for example, be performed on ABI 377 automated DNA sequencers using BigDye chemistry and cycle sequencing. Analysis of the sequencing runs will be limited to the 30-40 bases pinpointed by the T4E7 procedure as containing the variance. This provides the rapid identification of the altered base or bases.

20   In some cases, the presence of variances can be inferred from published articles which describe Restriction Fragment Length Polymorphisms (RFLP). The sequence variances or polymorphisms creating those RFLPs can be readily determined using convention techniques, for example in the following manner. If the RFLP was initially discovered by

25   the hybridization of a cDNA, then the molecular sequence of the RFLP can be determined by restricting the cDNA probe into fragments and separately hybridizing to a Southern blot consisting of the restriction digestion with the enzyme which reveals the polymorphic site, identifying the sub-fragment which hybridizes to the polymorphic restriction fragment, obtaining a

30   genomic clone of the gene (e.g., from commercial services such as Genome Systems (Saint Louis, Missouri) or Research Genetics (Alabama) which will provide appropriate genomic clones on receipt of appropriate primer pairs). Using the genomic clone, restrict the genomic clone with the restriction enzyme which revealed the polymorphism and isolate the fragment which

35   contains the polymorphism, e.g., identifying by hybridization to the cDNA which detected the polymorphism. The fragment is then sequenced across the polymorphic site. A copy of the other allele can be obtained by PCT from addition samples.

Variance detection using sequence scanning

In addition to the physical methods, e.g., those described above and others known to those skilled in the art (see, e.g., Housman, U.S. Patent 5,702,890; Housman et al., U.S. Patent Application 09/045,053), variances

5   can be detected using computational methods, involving computer comparison of sequences from two or more different biological sources, which can be obtained in various ways, for example from public sequence databases. The term "variance scanning" refers to a process of identifying sequence variances using computer-based comparison and analysis of

10   multiple representations of at least a portion of one or more genes. Computational variance detection involves a process to distinguish true variances from sequencing errors or other artifacts, and thus does not require perfectly accurate sequences. Such scanning can be performed in a variety of ways as known to those skilled in the art, preferably, for example, as

15   described in Stanton and Adams, U.S. Patent Application filed April 26, 1999, serial number not yet assigned, attorney docket 241/034.

While the utilization of complete cDNA sequences is highly preferred, it is also possible to utilize genomic sequences. Such analysis may be desired where the detection of variances in or near splice sites is sought. Such sequences may

20   represent full or partial genomic DNA sequences for a gene or genes. Also, as previously indicated, partial cDNA sequences can also be utilized although this is less preferred. As described below, the variance scanning analysis can simply utilize sequence overlap regions, even from partial sequences. Also, while the present description is provided by reference to DNA, e.g., cDNA, some sequences may be

25   provided as RNA sequences, e.g., mRNA sequences. Such RNA sequences may be converted to the corresponding DNA sequences, or the analysis may use the RNA sequences directly.

B.   Determination of Presence or Absence of Known Variances

30   The identification of the presence of previously identified variances in cells of an individual, usually a particular patient, can be performed by a number of different techniques as indicated in the Summary above. Such methods include methods utilizing a probe which specifically recognizes the presence of a particular nucleic acid or amino acid sequence in a sample. Common types of probes include

35   nucleic acid hybridization probes and antibodies, for example, monoclonal antibodies, which can differentially bind to nucleic acid sequences differing in one or more variance sites or to polypeptides which differ in one or more amino acid residues as a result of the nucleic acid sequence variance or variances. Generation

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and use of such probes is well-known in the art and so is not described in detail herein.

Preferably, however, the presence or absence of a variance is determined using nucleotide sequencing of a short sequence spanning a previously identified variance site. This will utilize validated genotyping assays for the polymorphisms previously identified. Since both normal and tumor cell genotypes can be measured, and since tumor material will frequently only be available as paraffin embedded sections (from which RNA cannot be isolated), it will be necessary to utilize genotyping assays that will work on genomic DNA. Thus PCR reactions will be designed, optimized, and validated to accommodate the intron exon structure of each of the genes. If the gene structure has been published (as it has for some of the listed genes), PCR primers can be designed directly. However, if the gene structure is unknown, the PCR primers may need to be moved around in order to both span the variance and avoid exon-intron boundaries. In some cases one-sided PCR methods such as bubble PCR (Ausubel et al. 1997) may be useful to obtain flanking intronic DNA for sequence analysis.

Using such amplification procedures, the standard method used to genotype normal and tumor tissues will be DNA sequencing. PCR fragments encompassing the variances will be cycle sequenced on ABI 377 automated sequencers using Big Dye chemistry

C.   Correlation of the Presence or Absence of Specific Variances with Differential Treatment Response

Prior to establishment of a diagnostic test for use in the selection of a treatment method or elimination of a treatment method, the presence or absence of one or more specific variances in a gene or in multiple genes is correlated with a differential treatment response. (As discussed above, usually the existence of a variable response and the correlation of such a response to a particular gene is performed first.) Such a differential response can be determined using prospective and/or retrospective data. Thus, in some cases, published reports will indicate that the course of treatment will vary depending on the presence or absence of particular variances. That information can be utilized to create a diagnostic test and/or incorporated in a treatment method as an efficacy or safety determination step.

Usually, however, the effect of one or more variances is separately determined. The determination can be performed by analyzing the presence or absence of particular variances in patients who have previously been treated with a particular treatment method, and correlating the variance presence or absence with the observed course, outcome, and/or development of adverse events in those

patients. This approach is useful in cases where both the observation of treatment effects was clearly recorded and cell samples are available or can be obtained. Alternatively, the analysis can be performed prospectively, where the presence or absence of the variance or variances in an individual is determined and the course,

5   outcome, and/or development of adverse events in those patients is subsequently or concurrently observed and then correlated with the variance determination.

Analysis of Haplotypes Increases Power of Genetic Analysis

Usually, variation in activity due to a single gene or a single genetic variance

10   in a single gene is not sufficient to account for observed variation in patient response to a treatment, e.g., a drug, there are often other factors that account for some of the variation in patient response. This is to be expected as drug response phenotypes usually vary continuously, and such (quantitative) traits are typically influenced by a number of genes (Falconer and Mackay, 1997). Although it is impossible to

15   determine *a priori* the number of genes influencing a quantitative trait, often only a few loci have large effects, where a large effect is 5-20% of total variation in the phenotype (Mackay, 1995).

Having identified genetic variation in enzymes that may affect action of a

20   specific drug, it is useful to efficiently address its relation to phenotypic variation. The sequential testing for correlation between phenotypes of interest and single nucleotide polymorphisms may be adequate to detect associations if there are major effects associated with single nucleotide changes; certainly it is useful to this type of analysis. However there is no way to know in advance whether there are major

25   phenotypic effects associated with single nucleotide changes and, even if there are, there is no way to be sure that the salient variance has been identified by screening cDNAs. A more powerful way to address the question of genotype-phenotype correlation is to assort genotypes into haplotypes. (A haplotype is the cis arrangement of polymorphic nucleotides on a particular chromosome.) Haplotype

30   analysis has several advantages compared to the serial analysis of individual polymorphisms at a locus with multiple polymorphic sites.

(1)   Of all the possible haplotypes at a locus (2^n haplotypes are theoretically possible at a locus with n binary polymorphic sites) only a small

35   fraction will generally occur at a significant frequency in human populations. Thus, association studies of haplotypes and phenotypes will involve testing fewer hypotheses. As a result there is a smaller probability of Type I errors, that is, false inferences that a particular variant is associated with a given phenotype.

(2)   The biological effect of each variance at a locus may be different both in magnitude and direction. For example, a polymorphism in the 5' UTR may affect translational efficiency, a coding sequence polymorphism may affect protein
5   activity, a polymorphism in the 3' UTR may affect mRNA folding and half life, and so on. Further, there may be interactions between variances: two neighboring polymorphic amino acids in the same domain - say cys/arg at residue 29 and met/val at residue 166 - may, when combined in one sequence, for example, 29cys-166val, have a deleterious effect, whereas 29cys-166met, 29arg-166met and 29arg-166val
10 proteins may be nearly equal in activity. Haplotype analysis is the best method for assessing the interaction of variances at a locus.

(3)   Templeton and colleagues have developed powerful methods for assorting haplotypes and analyzing haplotype/phenotype associations (Templeton et
15 al., 1987). Alleles which share common ancestry are arranged into a tree structure (cladogram) according to their time of origin in a population. Haplotypes that are evolutionarily ancient will be at the center of the branching structure and new ones (reflecting recent mutations) will be represented at the periphery, with the links representing intermediate steps in evolution. The cladogram defines which
20 haplotype-phenotype association tests should be performed to most efficiently exploit the available degrees of freedom, focusing attention on those comparisons most likely to define functionally different haplotypes (Haviland et al., 1995). This type of analysis has been used to define interactions between heart disease and the apolipoprotein gene cluster (Haviland et al 1995) and Alzheimer's Disease and the
25 Apo-E locus (Templeton 1995) among other studies, using populations as small as 50 to 100 individuals.

Methods for determining haplotypes
        The goal of haplotyping will be to identify the common haplotypes at
30 selected loci that have multiple sites of variance. Haplotypes will usually be determined at the cDNA level. Two general approaches to identification of haplotyes will be employed. First, haplotypes will be inferred from the pattern of allele segregation in families collected by the Centre d'Etude Polymorphisme Humaine. Cell lines from these families are available from the Coriell Repository.
35 Cell lines for all members of families 884, 102, 104 and 1331 are currently utilized. Cell lines from six additional families will also be used to increase the likelihood of detecting common haplotypes. This approach will be useful for cataloging common haplotypes and for validating methods on samples with known haplotypes. Second,

haplotypes will be determined directly from cDNA using the T4E7 procedure. T4E7 cleaves mismatched heteroduplex DNA at the site of the mismatch. If a heteroduplex contains only one mismatch, cleavage will result in the generation of two fragments. However, if a single heteroduplex (allele) contains two mismatches,

5   cleavage will occur at two different sites resulting in the generation of three fragments. The appearance of a fragment whose size corresponds to the distance between the two cleavage sites is diagnostic of the two mismatches being present on the same strand (allele). Thus, T4E7 can be used to determine haplotypes in diploid cells.

10   An alternative method, allele specific PCR, may be used for haplotyping. The utility of allele specific PCR for haplotyping has already been established (Michalatos-Beloin et al., 1996; Chang et al. 1997). Opposing PCR primers are designed to cover two sites of variance (either adjacent sites or sites spanning one or more internal variances). Two versions of each primer are synthesized, identical to

15   each other except for the 3' terminal nucleotide. The 3' terminal nucleotide is designed so that it will hybridize to one but not the other variant base. PCR amplification is then attempted with all four possible primer combinations in separate wells. Because Taq polymerase is very inefficient at extending 3' mismatches, the only samples which will be amplified will be the ones in which the

20   two primers are perfectly matched for sequences on the same strand (allele). The presence or absence of PCR product allows haplotyping of diploid cell lines. At most two of four possible reactions should yield products. This procedure has been successfully applied, for example, to haplotype the DPD amino acid polymorphisms.

25   D.   Selection of Treatment Method Using Variance Information
       1.   General
             Once the presence or absence of a variance or variances in a gene or genes is shown to correlate with the efficacy or safety of a treatment method, that information can be used to select an appropriate treatment method for a particular

30   patient. In the case of a treatment which is more likely to be effective when administered to a patient who has at least one copy of a gene with a particular variance or variances (in some cases the correlation with effective treatment is for patients who are homozygous for variance or set of variances in a gene) than in patients with a different variance or set of variances, a method of treatment is

35   selected (and/or a method of administration) which correlates positively with the particular variance presence or absence which provides the indication of effectiveness. As indicated in the Summary, such selection can involve a variety of different choices, and the correlation can involve a variety of different types of

treatments, or choices of methods of treatment. In some cases, the selection may include choices between treatments or methods of administration where more than one method is likely to be effective, or where there is a range of expected effectiveness or different expected levels of contra-indication or deleterious effects.

5   In such cases the selection is preferably performed to select a treatment which will be as effective or more effective than other methods, while having a comparatively low level of deleterious effects. Similarly, where the selection is between method with differing levels of deleterious effects, preferably a method is selected which has low such effects but which is expected to be effective in the patient.

10   Alternatively, in cases where the presence or absence of the particular variance or variances is indicative that a treatment or method of administration is more likely to be ineffective or contra-indicated in a patient with that variance or variances, then such treatment or method of administration is generally eliminated for use in that patient.

15

2.   Diagnostic Methods

Once a correlation between the presence and absence of at least one variance in a gene or genes and an indication of the effectiveness of a treatment, the determination of the presence or absence of that at least one variance provides

20   diagnostic methods, which can be used as indicated in the Summary above to select methods of treatment, methods of administration of a treatment, methods of selecting a patient or patients for a treatment. and others aspects in which the determination of the presence or absence of those variances provides useful information for selecting or designing or preparing methods or materials for medical

25   use in the aspects of this invention. As previously stated, such variance determination or diagnostic methods can be performed in various ways as understood by those skilled in the art.

In certain variance determination methods, it is necessary or advantageous to amplify one or more nucleotide sequences in one or more of the genes identified

30   herein. Such amplification can be performed by conventional methods, e.g., using polymerase chain reaction (PCR) amplification. Such amplification methods are well-known to those skilled in the art and will not be specifically described herein. For most applications relevant to the present invention, a sequence to be amplified includes at least one variance site, which is preferably a site or sites which provide

35   variance information indicative of the effectiveness of a method of treatment or method of administration of a treatment, or effectiveness of a second method of treatment which reduces a deleterious effect of a first treatment method, or which enhances the effectiveness of a first method of treatment. Thus, for PCR, such

amplification generally utilizes primer oligonucleotides which bind to or extent through at least one such variance site under amplification conditions.

For convenient use of the amplified sequence, e.g., for sequencing, it is beneficial that the amplified sequence be of limited length, but still long enough to
5   allow convenient and specific amplification. Thus, preferably the amplified sequence has a length as described in the Summary.

Also, in certain variance determination, it is useful to sequence one or more portions of a gene or genes, in particular, portions of the genes identified in this disclosure. As understood by persons familiar with nucleic acid sequencing. In
10 particular, sequencing can utilize dye termination methods and mass spectrometric methods. The sequencing generally involves a nucleic acid sequence which includes a variance site as indicated above in connection with amplification. Such sequencing can directly provide determination of the presence or absence of a particular variance or set of variances, e.g., a haplotype, by inspection of the
15 sequence (visually or by computer). Such sequencing is generally conducted on PCR amplified sequences in order to provide sufficient signal for practical or reliable sequence determination.

Likewise, in certain variance determinations, it is useful to utilize a probe or probes. As previously described, such probes can be of a variety of different types.
20

IV. Pharmaceutical Compositions, Including Pharmaceutical Compositions Adapted to be Preferentially Effective in Patients Having Particular Genetic Characteristics

1.   General
25 The methods of the present invention, in many cases will utilize conventional pharmaceutical compositions, but will allow more advantageous and beneficial use of those compositions due to the ability to identify patients who are likely to benefit from a particular treatment or to identify patients for whom a particular treatment is less likely to be effective
30 or for whom a particular treatment is likely to produce undesirable or intolerable effects. However, in some cases, it is advantageous to utilize compositions which are adapted to be preferentially effective in patients who possess particular genetic characteristics, i.e., in whom a particular variance or variances in one or more genes is present or absent (depending on whether
35 the presence or the absence of the variance or variances in a patient is correlated with an increased expectation of beneficial response). Thus, for example, the presence of a particular variance or variances may indicate that a patient can beneficially receive a significantly higher dosage of a drug than

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a patient having a different variance or variances.

2.   Regulatory Indications and Restrictions

The sale and use of drugs and the use of other treatment methods usually are

5   subject to certain restrictions by a government regulatory agency charged with
ensuring the safety and efficacy of drugs and treatment methods for medical use, and
approval is based on particular indications. In the present invention it is found that
variability in patient response or patient tolerance of a drug or other treatment often
correlates with the presence or absence of particular variances in particular genes.

10   Thus, it is expected that such a regulatory agency may indicate that the approved
indications for use of a drug with a variance-related variable response or toleration
include use only in patients in whom the drug will be effective, and/or for whom the
administration of the drug will not have intolerable deleterious effects, such as
excessive toxicity or unacceptable side-effects. Conversely, the drug may be given

15   for an indication that it may be used in the treatment of a particular disease or
condition where the patient has at least one copy of a particular variance, variances,
or variant form of a gene. Even if the approved indications are not narrowed to such
groups, the regulatory agency may suggest use limited to particular groups or
excluding particular groups or may state advantages of use or exclusion of such

20   groups or may state a warning on the use of the drug in certain groups. Consistent
with such suggestions and indications, such an agency may suggest or recommend
the use of a diagnostic test to identify the presence or absence of the relevant
variances in the prospective patient. Such diagnostic methods are described in this
description. Generally, such regulatory suggestion or indication is provided in a

25   product insert or label, and is generally reproduced in references such as the
Physician's Desk Reference (PDR). Thus, this invention also includes drugs or
pharmaceutical compositions which carry such a suggestion or statement of
indication or warning or suggestion for a diagnostic test, and which may also be
packaged with an insert or label stating the suggestion or indication or warning or

30   suggestion for a diagnostic test.

In accord with the possible variable treatment responses, an indication or
suggestion can specify that a patient be heterozygous, or alternatively, homozygous
for a particular variance or variances or variant form of a gene. Alternatively, an
indication or suggestion may specify that a patient have no more than one copy, or

35   zero copies, of a particular variance, variances, or variant form of a gene.

A regulatory indication or suggestion may concern the variances or variant
forms of a gene in normal cells of a patient and/or in cells involved in the disease or
condition. For example, in the case of a cancer treatment, the response of the cancer

cells can depend on the form of a gene remaining in cancer cells following loss of heterozygosity affecting that gene. Thus, even though normal cells of the patient may contain a form of the gene which correlates with effective treatment response, the absence of that form in cancer cells will mean that the treatment would be less

5   likely to be effective in that patient than in another patient who retained in cancer cells the form of the gene which correlated with effective treatment response. Those skilled in the art will understand whether the variances or gene forms in normal or disease cells are most indicative of the expected treatment response, and will generally utilize a diagnostic test with respect to the appropriate cells. Such a cell

10 type indication or suggestion may also be contained in a regulatory statement, e.g., on a label or in a product insert.

3.   Preparation and Administration of Drugs and Pharmaceutical Compositions Including Pharmaceutical Compositions Adapted to be Preferentially

15 Effective in Patients Having Particular Genetic Characteristics

A particular compound useful in this invention can be administered to a patient either by itself, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s). In treating a patient exhibiting a disorder of interest, a therapeutically effective amount of a agent or agents such as these is administered.

20 A therapeutically effective dose refers to that amount of the compound that results in amelioration of one or more symptoms or a prolongation of survival in a patient.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the

25 dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies

30 preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a

35 dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See *e.g.* Fingl et. al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p.1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or

5 adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of disorder of interest will vary with the severity of the condition to be treated and the route of administration. The severity

10 of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be

15 formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as

20 intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal

25 administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier

30 and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be

35 formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents

may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external

5   microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

            Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to

10   achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into

15   preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping or

20   lyophilizing processes.

            Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or

25   synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly

30   concentrated solutions.

            Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as

35   sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired,

disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone,
agar, or alginic acid or a salt thereof such as sodium alginate. Dragee cores are
provided with suitable coatings. For this purpose, concentrated sugar solutions may
be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone,

5   carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and
suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added
to the tablets or dragee coatings for identification or to characterize different
combinations of active compound doses.

    Pharmaceutical preparations which can be used orally include push-fit

10   capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a
plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active
ingredients in admixture with filler such as lactose, binders such as starches, and/or
lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft
capsules, the active compounds may be dissolved or suspended in suitable liquids,

15   such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition,
stabilizers may be added.

EXAMPLES

Example 1   Gene Identification

20 *Metabolic Pathways that Affect 5-FU/FA Action*

    The biochemical pathways of 5-FU metabolism have been studied
extensively. Likewise, folate metabolism has been well investigated and the
enzymes that form and consume 5,10-methylenetetrahydrofolate are well known.
The principal metabolic pathways that influence the pharmacologic action of 5-FU

25 are summarized below.

*De novo and salvage routes of pyrimidine nucleotide formation (5-FU
anabolism) and inhibition of thymidylate synthase*

    5-FU is a biologically inactive pyrimidine analog which must be

30 phosphorylated and ribosylated to the nucleoside analog fluorodeoxyuridine
monophosphate (FdUMP) to have clinical activity. FdUMP formation can occur via
several routes, summarized in Figure 1. 5-FU may be converted by uridine
phosphorylase to fluorouridine (FUdR; the reverse reaction is catalyzed by uridine
nucleosidase) and then to fluorouridine monophosphate (FUMP) by uridine kinase,

35 or FUMP may be formed from 5-FU in one step via transfer of a phosphoribosyl
group from 5-phosphoribosyl-1-pyrophosphate (PRPP), catalyzed by orotate
phosphoribosyl transferase. FUMP can be converted to FUDP and subsequently
FUTP by a nucleoside monophosphate kinase and nucleoside diphosphate kinase,
respectively. FUTP is incorporated into RNA by RNA polymerases, which may

account in part for 5-FU toxicity as a result of effects on processing or function (e.g.
translation). Alternatively, FUDP may be reduced to the dinucleotide level, FdUDP
(fluorodeoxyuridine diphosphate) by ribonucleotide diphosphate reductase, a
heterodimeric enzyme. FdUDP can then be converted to FdUTP by nucleoside
5   diphosphate kinase and incorporated into DNA by DNA polymerases which may
account for some 5-FU toxicity. Fluoropyrimidine modified DNA may also be
targeted by the nucleotide excision repair process. The more important path of
FdUDP metabolism with respect to anticancer effects, however, is believed to be
conversion to FdUMP by nucleoside diphosphatase (or cytidylate kinase, a
10   bidirectional enzyme). dUMP is the precursor of dTMP in de novo pyrimidine
biosynthesis, a reaction catalyzed by thymidylate synthase and which consumes
5,10-methylenetetrahydrofolate, producing 7,8 dihydrofolate. FdUMP, however,
forms an inhibitory (probably covalent) complex with thymidylate synthase in the
presence of 5,10-methylenetetrahydrofolate, thereby blocking formation of
15   thymidylate (other than by the salvage pathway via thymidine kinase). The complex
anabolism of FdUMP can be simplified by giving the deoxyribonucleoside of 5-FU,
5-fluorodeoxyuridine (also called floxuridine; FUdR), which can be converted to
FdUMP in one step by thymidine kinase. However, FUdR is also rapidly converted
back to 5-FU by the bidirectional enzyme thymidine phophorylase.
20

5-FU catabolism.

        Metabolic elimination of 5-FU occurs via a three step pathway leading to β-
alanine. The first and rate limiting enzyme in the elimination pathway is
dihydropyrimidine dehydrogenase (DPD), which transforms more than 80% of a
25   dose of 5-FU to the inactive dihydrofluorouracil form. Subsequently
dihydropyrimidinase catalyzes opening of the pyrimidine ring to form 5-fluoro-β-
ureidopropionate and then β-ureidopropionase (also called β-alanine synthase)
catalyzes formation of 2-fluoro-β-alanine. The first two reactions are reversible.
        The distribution of activity of these enzymes in human populations has not
30   been established, however, a recent population survey of urinary pyrimidine levels in
1,133 adults revealed that levels of dihydrouracil range from 0 - 59 uM/g of
creatinine, while uracil levels ranged from 0 - 130 uM/g creatinine (Hayashi et al.,
1996), suggesting variation in the activity of enzymes of pyrimidine metabolism. It
is worth noting that in animal studies catabolites of 5-FU apparently account for
35   some fraction of 5-FU toxicity (Davis et al., 1994; Spector et al., 1995). This result
is the rationale for current human trials of 5-FU combined with DPD inhibitors: if
the 5-fluoro- metabolites are responsible for toxicity, then blocking their formation
by inhibition of DPD, while simultaneously decreasing 5-FU dosage to compensate

for the block in catabolism and excretion, should result in a better therapeutic index.

Folinic acid conversion to tetrahydrofolate.

The conversion of FA to 5,10MTHF can occur via several routes, illustrated
5   in Figure 2.

Intracellular reduced folate levels can potentiate 5-FU action by increasing 5,10-methylenetetrahydrofolate levels (5,10-methyleneTHF; see center of Figure 2), thereby stabilizing the ternary inhibitory complex formed with thymidylate synthase and FdUMP. This is the basis for therapeutic modulation of 5-FU with FA. As can
10   be seen in Figure 2, conversion of folinic acid (5-formylTHF) to 5,10-methenylTHF, the precursor of 5,10-methyleneTHF, requires methenyltetrahydrofolate synthetase (enzyme 2 in the Figure). Also, levels of 5,10-methyleneTHF may be affected directly by the activity of methyleneltetrahydrofolate dehydrogenase, methyleneltetrahydrofolate reductase, serine transhydroxymethylase and the glycine
15   cleavage system enzymes (7, 8, 10 and 11 in Fig. 2), and indirectly by the other enzymes shown in the Figure.

Cell uptake of pyrimidine nucleosides and folinic acid

Human cells have five concentrative nucleoside transporters with varying
20   patterns of tissue distribution (see review by Wang et al., 1997). Two transporters, one with preference for purines and one for pyrimidines have been cloned recently (Felipe et al., 1998). 5-FU entry into cells may be modulated by activity of these transporters, particularly the pyrimidine transporter, although one prospective randomized clinical trial in which the nucleoside transport inhibitor dipyramidole
25   was paired with 5-FU and FA failed to show a difference in outcome compared to 5-FU/FA alone (Kohne et al., 1995). Several folate transport systems have been identified in human cells. Folate receptor 1 (FR1) is a high affinity (nanomolar range) receptor for reduced folates. Three restriction fragment length polymorphisms (RFLPs) have been reported at the FR1 locus (Campbell et al., 1991). Reduced
30   folates are also transported by folate receptor gamma and by a low affinity (1 uM) folate transporter. 15-fold variation in levels of folate transporter have been described in unselected tumor cell lines (Moscow et al., 1997).

Catalog allelic variation in enzymes that affect 5-FU and FA action
35   *Select genes for analysis of sequence variation*

In accord with the pathway description above, variation in either expression levels or intrinsic activity of the proteins involved in (i) cellular uptake of pyrimidines or reduced folate, (ii) conversion of 5-FU to the nucleotide form FdUMP, FUTP or FdUTP, (iii) catabolism of 5-FU, (iv) conversion of folinic acid to

5,10-methylenetetrahydrofolate or (iv) depletion of cellular 5,10-methylenetetrahydrofolate may be causally related to variation in clinical effect of 5-FU/FA. Table 3 below lists exemplary genes that will be, or already have been screened for polymorphism.

5

Table 2

Folate Transport	5-FU Anabolism	5-FU Catabolism	Conversion of Folinic Acid to 5,10-MethyleneTHF
Folate receptor 1 (α) GenBank M28099	Uridine phosphorylase GenBank X90858	*Dihydropyrimidine Dehydrogenase* GenBank U09178	Methylenetetrahydrofolate synthase GenBank L38298
Folate receptor (β) GenBank J02876	Thymidine phosphorylase GenBank S72487	Dihydropyrimidinase GenBank D78011	Methenyltetrahydrofolate cyclohydrolase; formyltetrahydrofolate synthetase; Methenyltetrahydrofolate dehydrogenase (one locus) GenBank J04031
Folate Transporter (SLC19A1) GenBank U19720	Orotate phosphoribosyl-transferase GenBank J03626	**Inhibition of dTMP Synthesis**	*Methylenetetrahydrofolate reductase* GenBank U09806
Folate receptor (γ) GenBank Z32564	Uridine Kinase GenBank D78335	*Thymidylate synthase* GenBank X02308	Serine transhydroxymeth-ylase 1 GenBank L11931
	Thymidine kinase 1 GenBank K02581; Thymidine Kinase 2 GenBank U77088		*Methionine synthetase* GenBank U50929
Pyrimidine Transport	Ribonucleoside reduct-ase: *M1 subunit* GenBank X59543 M2 subunit GenBank X59618	**Folate Polyglutamation**	Glycine cleavage system, Protein H:GenBank M69175; Protein P: GenBank M64590; Protein T: GenBank D13811
Nucleoside transporter 1	Nucleoside diphosphate kinase, A subunit GenBank U29200	Folylpolyglutamate synthetase GenBank M98045	*Dihydrofolate reductase* GenBank J00140
	B subunit GenBank X58965	Folylpolyglutamate hydrolase GenBank	

There are 27 genes in the above Table. Six genes which have already been surveyed for polymorphism are italicized. The following genes do not appear in the Table because there is no human cDNA in GenBank: 5-FU anabolism: Uridine monophosphate kinase; 5-FU catabolism: b-ureidopropionase; Folate metabolism: Glutamate formiminotransferase, Formiminotetrahydrofolate cyclodeaminase, Formyltetrahydrofolate hydrolase, Formyltetrahydrofolate dehydrogenase, and Protein L of the glycine cleavage system. Other genes not listed in the Table include DNA and RNA polymerases and DNA repair enzymes, some of which (e.g. DNA polymerase b and RNA polymerase II 220 and 33 kD subunits) have already been screened for polymorphism. Those additional genes are also useful in the present invention.

20

For several potential candidate genes there are mammalian cDNAs in GenBank but no human cDNA. For example, there is a 1,420 nucleotide full length rat β-ureidopropionase cDNA. Four overlapping human ESTs (F06711, H19181, R11806 and W55897) span 691 nucleotides of the rat coding sequence with >90% nucleotide identity. For selected candidate genes of likely importance, such as β-ureidopropionase, polymorphism analysis will be carried out on the available human sequence from dbEST.

Example 2 Variance Identification - Variances in Genes That Can Affect 5-FU/FA Action

Exemplary genes related to modulation of the action of 5-FU/FA have been analyzed for genetic variation; thymidylate synthase, ribonucleotide reductase (M1 subunit only), dihydrofolate reductase and dihydropyrimidine dehydrogenase cDNAs. 36 unrelated individuals were screened using 6 SSCP conditions and DNA sequencing. Other investigators have identified variances in MTHFR, methionine synthase and folate receptor. These findings are summarized in Table 3.

Table 3: Variation in Genes Which Modulate 5-FU/FA Pharmacology

Gene Name (Genbank accession no.)	Variances			Heterozy-gote Frequency	Comments
	Base	RNA	Protein		
Cytidine Deaminase (L27943)	79	T or G	lys27glu	>10%	
Dihydrofolate Reductase (J00140)	721 829 RsaI RFLP ScrF1 RsaI RFLP	T or A C or T		20% 14% 23, 33, 43% 26% 32%	3 alleles unique RsaI RFLP
Dihydropyrimidinase (D78011)	1001 1303 203 1468 1078 812 to 814	A or G G or A G or C G or C T or C Insertion A	gln334arg gly435arg thr68arg arg490thr trp360arg premat. term.	rare rare	All found in patients with DHP deficiency

Dihydropyrimidine Dehydrogenase (U09178)	166	T or C	cys29arg	11%	
	577	A or G	met166val	9%	
	3925	A or G	3' UTR	35%	
	3937	T or C	3' UTR	38%	
	3432	T or C	3' UTR	10%	
			arg21gln	rare	
			val335leu	rare	
	638	A or G	tyr186cys	2%	
	784	C or T	arg235trp	rare	
	296 to 299	Delete TCAT	premat. term.	rare	
	1682	G or A	ser534asn	0.5-3%	
	1708	A or G	ile543val	7-35%	
	exon/intron	G or A	del. 581-635	1%	73% in DPD deficiency
	14	delete C	premat. term.	rare	
	1897	G or A	val732ile	1-7%	
	2275	G or A	arg886his	rare	
	2738	A or T	asp974trp	rare	
	3002	G or T	val995phe	rare	
	2983				
Folate Receptor α (M28099)					One Msp I and 2 Pst I RFLPs
Folate receptor γ (Z32564)	330-331	2 bp deletion	Premat. Term.	75%	
Folate Transporter (SLC19A1) (U19720)	341	C or G	Silent	1%	
Folylpolyglutamate Synthetase (M98045)	1747	G or T	3' UTR	2%	
	1900	T or C	3' UTR	50%	
Glycine cleavage System: protein H (M69175)	710	C or G	3' UTR	7%	
Glycine cleavage System: protein P (M64590)			ser564ile	rare	70% in NKH patients
Glycine cleavage System: protein T (D13811)	277	G or T	Val50leu	2%	
	1073	G or A	Arg315lys	1%	
	1083	G or A	Silent	2%	
	1773	C or T	3'UTR	3%	
Methenyltetrahydro-folate cyclohydrolase	454	G or A	Arg134lys	22%	
	969	C or G	Gln306glu	1%	
	1614	C or T	Silent	1%	
	2011	G or A	Arg653gln	35%	
			Arg293his	rare	
Methylenetetra-hydrofolate Reductase (U09806)	129	C or T		Low	Both the amino acid changes affect MTHFR activity.
	677	C or T	Ala223val	48%	
	1068	C or T		low	
	1298	C or A	Ala430glu	high	
	308	T or C	silent	5-39%	
				rare	Rare mutations found in MTHFR deficiency
Methionine Synthase (U50929, U73338))	2756	G or A	Asp919gly	19-29%	Affects folate levels in colon cancer patients.
	3970	T or C	Silent		
	1158	G or A	Cys225try	rare	
	1004	G or T	Ala to ser		
				rare	Rare mutations found in MS deficiency
Nucleoside Diphosphate kinase B (X58965)			BgII RFLP		

Ribonucleotide Reductase, M1 (X59543)	1037	C or A		33%	
	2410	A or G		40%	
	2419	A or G		20%	
	2717	T or A		19%	
	2724	T in/del		19%	
			SacI RFLP	47%	
Ribonucleotide Reductase, M2 (X59618)	524	C or G	Silent	1%	
	1636	C or T	3' UTR	1%	
	2259	T or C	3' UTR	1%	
Serine Hydroxy-methyltransferase (cytolic) (L11931)	1444		Leu474phe	23%	
	1541	C or T	3' UTR	26%	
Thymidine kinase 1 (K02581)	90	T or C	Silent	50%	
	279	G or A	Silent	13%	
	282	G or A	Silent	30%	
	772	G or A	3' UTR	26%	
	867	G or A	3' UTR	50%	
			Tac1 RFLP	40%	
			BstEII RFLP	2, 34, 64 %	3 alleles
Thymidine kinase 2 (U77088)	1480	T or C	3' UTR	9%	
Thymidine Phosphorylase (PD-ECGF) (S72487)	601	G or C	3' UTR	3%	
	3673	A or G			
	3576	T or C	silent	54%	
				rare	Rare mutations found in MNGIE patients
Thymidylate Synthase (X02308)	276	T or C	tyr33his	rare	
	1140	C or T		53%	
	1210	A or G		42%	
	1571	A or T		53%	
	28-34 nt repeats		5' reg. Region	double: 19%	
Uridine mono-Phosphate synthetase (J03626)	742	G or C	Gly213ala	23%	
	1575	A or G	3' UTR	1%	
				rare	Rare mutations found in Orotic aciduria patients

A more complete catalog of genetic variances is shown in the following table for the dihydropyrimidine dehydrogenase (DPD) gene.

Table 4
Variances in Dihydropyrimidine Dehydrogenase Gene

Variant nucleotide (codon)	Variant base 1 (frequency)	Variant base 2 (frequency)	Effect on mRNA & protein	Comments
166 (29)	T (62/70)	C (8/70)	cys29arg	Arg allele has no activity when expressed in E. Coli (Vreken, Human Genetics, 1997)
577 (166)	A (69/72)	G (3/72)	met166val	Located in highly conserved domain; no functional studies
784 (235)	C	T	arg235trp	Trp allele has no activity when expressed in E. Coli (Vreken, Human Genetics, 1997)
1682 (534)	G (148/150)	A (2/150)	ser534asn	Apparently little or no functional effect in patient cells.
1708 (543)	A (34/46)	G (12/46)	ile543val	Apparently little or no functional effect in patient cells.
intron 13 (destroys 5' GT splice site	G	A	no exon 14	55 missing amino acids result in unstable protein. Mutant allele may be present in ~1% of Finns; very rare in other groups, but

immediately after nt 1986)				detected in 8 of 11 patients with complete deficiency.
1897 (606)	-	deletion of C	frameshift	Low/no activity allele; reported in only one patient so far.
2738 (886)	G	A	arg886his	His allele has ~25% of normal activity when expressed in Coli (Vreken, Human Genetics, '97)
3002 (974)	A	T	asp974val	Val allele apparently has very low or no activity in patient sample. Very low frequency allele (<0.2% in Americans).
3925	A (41/62)	G (21/62)	3' UTR	Two high frequency variances, 12 nt apart but not in complete
3937	C (40/64)	T (24/64)	3' UTR	linkage disequilibrium.

Variances in the exemplary genes above which affect the activity of the corresponding gene product have the potential to modulate the activity of 5-FU/FA

5   and thereby provide predictive capability concerning the efficacy of such treatment in a particular patient. As discussed above, such predictive capability can further be provided by the joint determination of multiple variances, in one or a plurality of genes or both. Similarly, such variances can provide such predictive capability for other treatments, e.g., treatments with other compounds, which involve these genes.

10

Example 3   Relationship of Genes to Drug Response – 5-flurouracil

5-fluorouracil (5-FU) is a widely used chemotherapy drug. The effectiveness of 5-FU is potentiated by folinic acid (FA; generic name: leukovorin). The

15   combination of 5-FU and FA is standard therapy for stage III/IV colon cancer. Patient responses to 5-FU and 5-FU/FA vary widely, ranging from complete remission of cancer to severe toxicity.

Clinical Use and Effectiveness of 5-FU and 5-FU/FA

20   5-FU is a pyrimidine analog in clinical use since 1957. 5-FU is used in the standard treatment of gastrointestinal, breast and head and neck cancers. Clinical trials have also shown responses in cancer of the bladder, ovary, cervix, prostate and pancreas. The remainder of this discussion will concern colorectal cancer. 5-FU is used both in the adjuvant therapy of Dukes Stage B and C cancer and in the

25   treatment of disseminated cancer. 5-FU alone produces partial remissions in 10-30% of advanced colorectal cancers, however only a few percent of patients have complete remissions, and no benefit in survival has been demonstrated.

In the last 15 years a variety of biochemically motivated strategies for modulating 5-FU activity have been tested. For example, 5-FU has been used in

30   combination with PALA, a pyrimidine synthesis inhibitor, to deplete cellular pools

of UTP and thereby enhance formation of FUTP; in combination with methotrexate, to inhibit purine anabolism, leading to increased PRPP levels and consequent increased conversion of 5-FU to its active nucleotide metabolites; and in combination with folinic acid, which increases intracellular pools of reduced folate,

5   driving formation of the ternary inhibitory complex formed by 5,10 methylenetetrahydrofolate, FdUMP and thymidylate synthase. Levamisole, interferon and alkylating agents have also been used in combination with 5-FU. 5-FU/Levamisole and 5-FU/FA are widely used in the adjuvant treatment of colon cancer, while 5-FU/FA is the most commonly used regimen for advanced colorectal

10   cancer. Six of seven prospective randomized trials of 5-FU/FA vs. 5-FU alone in patients with advanced cancer have demonstrated up to two fold higher response rates to 5-FU/FA, while two of the studies also showed increased survival.

Two major dosing regimens are used: 5-FU plus low dose FA given for five consecutive days followed by a 23 day interval, or once weekly bolus iv 5-FU plus

15   high dose FA. The higher FA dose results in plasma FA concentrations of 1 to 10 uM, comparable to those required for optimal 5-FU/FA synergy in tissue culture, however low dose FA (20 mg/m² vs. 500 mg/m²) has produced comparable clinical benefit. Ongoing clinical trials are designed to further test new drug combinations. In summary, relatively few patients - in the single digits - live longer as a result of 5-

20   FU/FA, although significantly more have partial disease remission. The factors that determine which patients respond or have side effects are not known.

5-FU modulators

Leukovorin (folinic acid) is the most widely used 5-FU modulator, however

25   a variety of other molecules have been used with 5-FU, including, for example, interferon-alpha, hydroxyurea, N-phosphonacetyl-L-aspartate, dipyridamole, levamisole, methotrexate, trimetrexate glucuronate, cisplatin and radiotherapy. S-1 is a novel oral anticancer drug, composed of the 5-FU prodrug tegafur plus gimestat (CDHP) and otastat potassium (Oxo) in a molar ratio of 1:0.4:1, with CDHP

30   inhibiting dihydropyrimidine dehydrogenase in order to prolong 5-FU concentrations in blood and tumour and Oxo present as a gastrointestinal protectant. Some of these regimens show promising results, but no clear improvement over 5-FU/leukovorin. The clinical development and use of regimens containing 5-FU plus modulators may be facilitated by the methods of this invention.

35

Toxicity of 5-FU and Folinic Acid

5-FU toxicity has been well documented in randomized clinical trials.

Patients receiving 5-FU/FA are at even greater risk of toxic reactions and must be monitored carefully during therapy. A variety of side effects have been observed, affecting the gastrointestinal tract, bone marrow, heart and CNS. The most common toxic reactions are nausea and anorexia, which can be followed by life threatening

5   mucositis, enteritis and diarrhea. Leukopenia is also a problem in some patients, particularly with the weekly dosage regimen. In a recent randomized trial of weekly vs. monthly 5-FU/FA, there were 7 deaths related to drug toxicity among 372 treated patients (1.9%; Buroker et al. 1994). 31% of patients receiving the weekly regimen suffered diarrhea requiring hospitalization for a median of 10 days. Other severe

10   toxicities, which occured at lower frequency, included leukopenia and stomatitis. In another example, 36% of patients receiving weekly bolus 5-FU plus FA (500 mg/m^2), in a NSABP trial suffered NCI grade 3 toxicity (Wolmark et al., 1996). Clearly, toxicity is a major cost of 5-FU/FA therapy, measured both in patient suffering and in financial terms (the cost of care for drug induced illness).

15

Other Factors

         Many non-genetic factors can influence the response of cancers to drugs, including tumor location, vasculature, cell growth fraction and various drug resistance mechanisms. It is therefore not possible to explain all heterogeneity in

20   response to 5-FU/FA administration by genetic variation. However, based on genetic studies of other quantitative traits it appears that a significant fraction of variation in drug response is due to genetic variation.

25   **Example 4**   Genetic Component of Drug Response Variability
     Genetically Determined Variation in Response to 5-FU: Studies of
     Dihydropyrimidine Dehydrogenase Deficiency

     Dihydropyrimidine Dehydrogenase Deficiency is Associated with 5-FU
30   *Toxicity*

         5-FU is inactivated by the same metabolic pathway as thymine and uracil (see above). DPD catalyzes the first, rate limiting step in pyrimidine catabolism and accounts for elimination of most 5-FU. Normal individuals eliminate 5-FU with a half life of ~10-15 minutes and excrete only 10% of a dose unchanged in the urine.

35   In contrast, people genetically deficient in DPD eliminate 5-FU with a half life of ~2.5 hours and excrete 90% of a dose unchanged in the urine (Diasio et al., 1988). DPD deficiency has two clinical presentations: (i) an inborn error of metabolism causing some degree of neurologic dysfunction or (ii) asymtomatic until revealed by exposure to 5-FU or other pyrimidine analogs. With either presentation there is

combined hyperuraciluria and hyperthyminuria. The vastly increased 5-FU half life in DPD deficient individuals causes severe toxicity and even death. Recently several mutations have been identified in DPD genes of deficient individuals (Wei et al., 1996), however none of these alleles appears to occur at appreciable frequency, so

5   the cause of wide population variation in DPD levels is still not understood.

Dihydropyrimidine dehydrogenase (DPD) inhibitors

More than 85% of an injected dose of 5-FU is rapidly inactivated by dihydropyrimidine dehydrogenase (DPD) to therapeutically inactive catabolic

10   products, however there is evidence that said catabolic products may be toxic to normal tissues. This has led to the development of DPD inhibitors with the aim to modify the therapeutic index of 5-FU. Several inhibitors in combination with 5-FU are under preclinical and clinical evaluation, including uracil and 5-chloro-2,4-dihydroxy pyridine, as modulators of 5-FU derived from its prodrug tegafur and 5-

15   ethynyluracil as a modulator of 5-FU itself (Eniluracil, 776C85; Glaxo Wellcome Inc, Research Triangle Park, NC). Other compounds with DPD inhibitory activity include 5-propynyluracil. (For a review of DPD inhibitors see: Diasio, RB Improving 5-FU with a Novel Dihydropyrimidine Dehydrogenase Inactivator, Oncology 1998, Mar; 12(3 Suppl. 4):51-6.)

20

Population Studies of DPD Activity Show Wide Variation

Population surveys of DPD activity in normal individuals have been performed using blood and liver samples. These studies reveal a broad unimodal Gaussian distribution of DPD activity over a 7 to 14 fold range, with some

25   individuals having very low or even undetectable levels. For example Etienne et al. (1994) report DPD activity ranging from .065 to .559 nM/min/mg protein in a study of 152 men and 33 women, while Fleming et al. (1993) found DPD activity in 66 cancer patients varied from .17 to .77 nM/min/mg protein. Lu et al (1995) found 18-fold variation in liver DPD assayed in 138 individuals. Milano and Etienne (1994)

30   suggested that the frequency of heterozygous and homozygous deficiendy is 3% and .1%, respectively. The DNA sequence alterations responsible for null DPD alleles do not account for the high population variability (Ridge et al., 1997).

DPD Levels Correlate with Response to 5-FU

35   Intratumoral DPD levels have been measured in patients receiving 5-FU chemotherapy. When complete responders were compared to partial or nonresponders, DPD levels were lower in the compete responders (Etienne et al., 1995). Leukocyte DPD levels have also been measured in patients receiving 5-FU/FA chemotherapy. When patients were divided into 3 groups: high, medium and

low DPD activity, the frequency of serious side effects was highest in the low DPD group and vice versa (Katona et al., 1997).

Biochemical Studies of Alternate Allelic Forms of DPD

The power of genetic analysis can be augmented by biochemical studies of alternate allelic forms of enzymes. Biochemical data on the distribution of activity of a series of enzymes in a biochemical pathway provides the basis for metabolic flux analysis (Keightly, 1996). It is beyond the scope of this proposal to exhaustively analyze biochemical variation in the enzymes of pyrimidine and folate metabolism. However, since we have identified new variances in DPD that may affect enzyme expression or activity, and because DPD is already proven to play a role in 5-FU response, we will determine the relationship between genotype and biochemistry for this enzyme.

DPD cDNAs have been cloned from a variety of higher eukaryotes and binding sites for its cofactors, prosthetic groups and substrate have been defined experimentally or by analogy with known consensus motifs (Yokata et al., 1994). The DPD polymorphisms that affect protein sequence occur at amino acids 29 (cys/arg) and 166 (met/val) in the amino-terminal one-third of the protein. Phylogenetic comparison of this region from boar, human, cow, fly, and bacteria (see below) shows that there are actually two highly conserved motifs that resemble either iron/sulfur or zinc binding motifs, the latter being more likely due to the spacing of the cysteine residues. The region around the met/val polymorphism at amino acid 166 is highly conserved. Even the spacing of the putative zinc-finger domains is maintained between distantly related species, hinting at their importance. Since amino acid 166 is close to a highly conserved (and probably functionally important) region and is itself conserved, being a methionine in all species, it seems likely that perturbations in this position would have consequence. The polymorphism substitutes a long amino acid side chain capable of hydrogen bonding (methionine) for a compact, hydrophobic amino acid (valine). The region around amino acid 29 is not as well conserved.

Common DPD Haplotypes

Eight haplotypes from 58 chromosomes (29 individuals) have been identified. Using methods described above, the DNA from these samples were analyzed by PCR. The single base pair substitutions at four locations were identified as allelic haplotypes, e.g. base pair number 166, 577, 3925, 3937. Base pair positions, 3925 and 3937 are located in the 3 prime untranslated region of the cDNA and base pairs 166 and 577 are within the coding region.

Table 5
Identified DPD Haplotypes

No. Chromosomes	Base Position			
	166	577	3925	3937
14 (24%)	T (cys)	A (met)	G	C
16 (28%)	T (cys)	A (met)	A	C
16 (28%)	T (cys)	A (met)	A	T
4 (7%)	C (arg)	A (met)	A	T
3 (5%)	C (arg)	A (met)	G	C
3 (5%)	C (arg)	A (met)	A	C
1 (2%)	T (cys)	G (val)	G	C
1 (2%)	T (cys)	G (val)	A	C
Total=58(100%)				

Example 5 – Exemplary Genes involved in Folate Transport and Metabolism

While examples above concern 5-FU/FA action and genes which are expected to modulate such action, it is also useful to utilize genes involved in folate transport and metabolism generally. A number of these genes are also involved in 5-FU/FA action. Genes known to be involved in folate transport and metabolism are listed in the table below, along with available GenBank accession numbers for deposited sequences.

Table 6
Gene Field: Folate Transport & Metabolism

Folate Transporters	Folate Polyglutamation	Biosynthesis, Degradation and Interconversion of Folates	
Folate receptor 1(α) (GenBank M28099)	Folylpolyglutamate synthetase (GenBank M98045)	Formiminotetrahy-drofolate cyclodeaminase	Glutamate form-iminotransferase
Folate receptor (β) (GenBank J02876)		Methenyltetrahy-drofolate synthetase	Formyltetrahydrofolate hydrolase
Folate receptor (γ) (GenBank Z32564)		Methylenetetrahy-drofolate dehydrogenase	Methylenetetrahydrofolate synthase GenBank L38298
Folate Transporter (SLC19A1) GenBank U19720		*Methionine synthetase* GenBank U50929	*Methylenetetrahydrofolate reductase* GenBank U09806
Folate Absorbtion	Inhibition of dTMP Synthesis	*Dihydrofolate reductase* GenBank J00140	Serine transhydroxy-methylase 1 GenBank L11931

Pteroyl-γ-glutamyl carboxypeptidase	*Thymidylate synthase* GenBank X02308	Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase (one locus) GenBank J04031	Glycine cleavage system, Protein H: GenBank M69175; Protein P: GenBank M64590; Protein T: GenBank D13811; Protein L . Formyltetrahydrofolate dehydrogenase

Genes affecting the action of drugs which modulate folate metabolism.
There are 24 genes in the Table, four of which we have already surveyed for
5   polymorphism (*italicized genes*). The genes with GenBank numbers are currently
being screened for variances. Genes lacking GenBank numbers are not yet
represented in GenBank as full length cDNAs; but will be scanned using relevant
EST collections or using sequences from other publicly available sources.

10

Example 6 – Drugs Targeting Genes Involved in Folate Transport and Metabolism
In concert with the identification of useful genes involved in folate transport
and metabolism, the table below identifies certain drug classes used for treatment of
identified disorders, along with a brief characterization of the action of the drug.
15   Exemplary drugs are identified within the individual classes. Variable response of
patients to administration of drugs of these classes, or administration of the specific
drugs can be used in identifying variances responsible for such variable response.
As described above, those variances can then be used in diagnostic tests, methods of
selecting a treatment, methods of treating a patient, or other methods utilizing
20   genetic variance information as otherwise described.

Table 7
Drug Field: Folate Transport & Metabolism

Disease/ Indication	Drug Class	Mechanism of Action	Exemplary Drugs
Cancer	Reduced folates	Block dTMP biosynthesis by inhib-iting thymidylate synthase (TS) via formation of ternary complex invol-ving TS, 5-fluorodeoxyuridine and 5,10-methylenetetrahydrofolate	leukovorin, L-leu-kovorin, citrovor-um factor (used with 5-fluorouracil or related drugs)
Cancer	Reduced folates	Rescue bone marrow from lethal toxicity after high dose methotrexate	leukovorin, L-leu-kovorin, citrovor-um factor
Cancer	Folate analogs (antifolates)	Block *de novo* purine biosynthesis by inhibiting dihydrofolate reduc-tase, TS,	Methotrexate, aminopterin, dide-azatetrahydrofolate
Proliferative skin diseases (psoriasis)	Folate analogs (antifolates)	Block *de novo* purine biosynthesis by inhibiting dihydrofolate reduc-tase, TS,	Methotrexate, aminopterin, dide-azatetrahydrofolate

Immunosup-pression	Folate analogs (antifolates)	Block *de novo* purine biosynthesis by inhibiting dihydrofolate reduc-tase, TS,	Methotrexate, aminopterin, dide-azatetrahydrofolate
Autoimmune diseases, such as rheumatoid arthritis	Folate analogs (antifolates)	Block *de novo* purine biosynthesis by inhibiting dihydrofolate reduc-tase, TS,	Methotrexate, aminopterin, dide-azatetrahydrofolate
Folate deficiency	Folic acid	Increase folates for purine and pyrimidine biosynthesis	Folic acid
Cardiovascular disease (prevent atherosclerosis)	Folic acid	Reduce plasma homocysteine levels in patients with low MTHFR levels	Folic acid
Prevent spina bifida	Folic acid	Reduce plasma homocysteine levels in patients with low MTHFR levels	Folic acid

Table 7. Drugs which affect or are affected by folate metabolism. A wide spectrum of diseases are treated with drugs that affect folate metabolism. Some drugs are used in the treatment of several diseases. All of the listed drugs are

5   frequently used in combination with other drugs. For example methotrexate is used in cancer chemotherapy with cytoxan and fluoruracil to treat breast cancer, among other combinations.

Folate analogs

10   Many novel antifolate compounds with unique pharmacologic properties are currently in clinical development. These newer antifolates differ from methotrexate, the most widely used and studied drug in this class, in terms of their lipophilicity, cellular transport mechanism, level of polyglutamation, and specificity for inhibiting folate-dependent enzymes, such as dihydrofolate reductase, thymidylate synthase, or

15   glycinamide ribonucleotide formyltransferase. The clinical development and use of these new compounds can be affected by the methods of this invention. The new folate analogs include quinazoline derivatives such as ZD1694 (Tomudex, AstraZeneca) which requires Reduced Folate Carrier (RFC) mediated cell uptake and polyglutamation by Folylpolyglutamate Synthetase (FPGS); ZD9331

20   (AstraZeneca), which requires the RFC but is not polyglutamated by FPGS; LY231514 (Eli Lilly Research Labs, Indianapolis, IN) is a multitargeted pyrrolopyrimidine analogue antifolate which requires the RFC and polyglutamation; GW1843 (1843U89, GlaxoWellcome) is a benzoquinazoline compound with potent TS inhibitory activity, and which enters cells via the RFC but is polyglutamated only

25   to the diglutamate, which leads to higher cellular retention without augmenting TS inhibitory activity; AG337 (p.o. and i.v. forms) and AG331 (both by Agouron, La Jolla, CA, now part of Warner Lambert) are lipophilic TS inhibitors with action independent of the RFC and polyglutamation by FPGS; trimetrexate (US Bioscience) is a ; Aminopterin is an older drug which has received renewed attention

30   recently; edatrexate, piritrexim and lometrexol are other antifolate drugs. More

generally, 5,8-dideazaisofolic acid (IAHQ), 5,10-dideazatetrahydrofolic acid (DDATHF), and 5-deazafolic acid are structures into which a variety of modifications have been introduced in the pteridine/quinazoline ring, the C9-N10 bridge, the benzoyl ring, and the glutamate side chain (see article below). Also

5   Lilly have recently synthesized a new series of 2,4-diaminopyrido[2,3-d]pyrimidine based antifolates which are being evaluated both as antineoplastic and antiarthritic agents.

Other Therapeutic Categories in which Folate or Pyrimidine Pathwyas may be
10   *Relevant to Drug Development*

       1) Cardiovascular Drugs

       Homocysteine is a proven risk factor for cardiovascular disease. One important role of the folate cofactor 5-methyltetrahydrofolate is the provision of a methyl group for the remethylation of homocysteine to methionine by the enzyme

15   methionine synthase. Variation in the enzymes of folate metabolism, for example methionine syntase or methylenetetrahydrofolate reductase (MTHFR), may affect the levels of 5-methyltetrahydrofolate or other folates that in turn influence homocysteine levels. The contribution of elevated homocysteine to atherosclerosis, thromboembolic disease and other forms of vascular and heart disease may vary

20   from one patient to another. Such variation may be attributable, at least in part, to genetically determined variation in the levels or function of the enzymes of folate metabolism described in this application. Assistance of clinical development or use of drugs to treat said cardiovascular diseases might be afforded by an understanding of which patients are most likely to benefit. This is true whether the drugs are aimed

25   at the modulation of folate levels (e.g. supplemental folate) or at other known causes of cardiovascular disease (e.g. lipid lowering drugs such as statins, or antithrombotic drugs such as salicylates, heparin or GPIIIa/IIb inhibitors). It may, for example, be desirable to exclude patients whose disease is significantly attributable to elevated homocysteine from treatment with agents aimed at the amelioration of other

30   etiological causes, such as elevated cholesterol. Thus, the understanding of variation in the enzymes of folate transport and metabolism may be important in evaluating drugs used to treat atherosclerosis, thromboembolic diseases and other forms of vascular and heart disease.

35   2) CNS drugs

       The observation that phencyclidine, an NMDA receptor antagonist, induces a psychotic state closely resembling schizophrenia in normal individuals has led to attempts to modulate NMDA receptor function in schizophrenic patients. The amino

acid glycine is an obligatory coagonist (with glutamate) at NMDA receptors (via its
action at a strychnine-insensitive binding site on the NMDA receptor complex), and
consequently glycine or glycinergic agents (e.g. glycine, the glycine receptor partial
agonist, D-cycloserine, or the glycine prodrug milacemide) have been tried as an
5   adjunct to conventional antipsychotics for the treatment of schizophrenia. Several
trials have demonstrated a moderate improvement in negative symptoms of
schizophrenia. Because the folate pathway modulates levels of serine and glycine,
the endogenous levels of glycine in neurons may affect the response to glycine or
glycinergic drugs. In particular, interpatient variation in glycine metabolism may
10   affect drug efficacy.

Example 7 – Genes Related to Pyrimidine Transport and Metabolism

15   Similar to the genes involved in folate transport and metabolism, genes
involved in the related pathways of pyrimidine transport and metabolism are useful
in the aspects of the present invention, e.g., for identifying variances responsible for
variable treatment response, diagnostic methods, and methods of selecting a patient
to receive a treatment. Exemplary genes are provided below and are further
20   identified by cellular function. Genes involved in those functions are generally
. useful in the present invention.

Table 8
Gene Field: Pyrimidine Transport & Metabolism

25

Pyrimidine Transport	Pyrimidine Biosynthesis - *de novo* and Salvage Pathways		Pyrimidine Catabolism
Equilibrative nucleoside transporter 1	Uridine phosphorylase GenBank X90858	Ribonucleoside reductase: *M1 subunit* GenBank X59543 M2 subunit GenBank X59618	*Dihydropyrimidine Dehydrogenase* GenBank U09178
Equilibrative nucleoside transporters 2, 3, 4 & 5	Thymidine phosphorylase GenBank S72487	Nucleoside diphosphate kinase, A subunit GenBank U29200	Dihydropyrimidinase GenBank D78011
Concentrative nucleoside transporters	Orotate phosphoribosyl-transferase GenBank J03626	B subunit GenBank X58965	β-ureidopropionase
	Uridine Kinase GenBank D78335	Uridine mono-phosphate kinase	Cytidine deaminase
	Thymidine kinase GenBank K02581; Thymidine Kinase 2 GenBank U77088	Deoxycytidylate kinase	dCMP deaminase

Inhibition of dTMP Synthesis	Deoxycytidine kinase			β-alanine-pyruvate aminotransferase
Thymidylate synthase GenBank X02308				β-alanine-α-detoglutarate aminotransferase

Table 8. Genes affecting the action of drugs which modulate pyrimidine metabolism. We have already surveyed three of the above genes for polymorphism (*italicized genes*). The genes with GenBank numbers are currently being screened for variances. Genes in the table lacking GenBank numbers are not yet represented in GenBank as full length cDNAs; but can be evaluated using relevant EST collections. Genes not listed in the Table but related to the mechanism of action of pyrimidine analogs include DNA and RNA polymerases and subunits and DNA repair enzymes, some of which (e.g. DNA polymerase β and 220 kD and 33 kD subunits of RNA polymerase II) have already been screened for polymorphism. Such additional genes can also be used in the present invention.

Example 8 – Drugs Targeting Genes Involved in Pyrimidine Transport & Metabolism

As was described above for drugs modulating genes involved in folate transport and metabolism, particular drug classes and exemplary drugs are identified in the table below which modulate the action of pyrimidine transport and metabolism genes. These classes of drugs and exemplary drugs are similarly useful for identifying variances which affect the action

Table 9
Drug Field: Pyrimidine Transport & Metabolism

Disease/ Indication	Drug Class	Mechanism of Action	Exemplary Drugs
Cancer	Fluoropyrimidines	Block dTTP biosynthesis by inhib-iting thymidylate synthase; inhibit replication, transcription and/or repair by incorporation into DNA and RNA.	5-FU, fluorode-oxyuridine, flu-orodeoxyuridine monophosphate, tegafur, florafur.
Cancer	Dihydropyrimidine dehydrogenase inhibitors	Potentiate fluoropyrimidines by blocking their catabolism, increasing half life.	5-ethynyluracil; 5-propynyluracil; 2,6 dihydroxypy-ridine
Cancer	Cytidine analogs	Incorporation into DNA and conse-quent inhibition of DNA synthesis (replication, transcription, repair).	Cytosine arabino-side, gemcitabine, 5-azacytidine, 5-azacytosine ara-binoside, others.
Cancer	Other pyrimidine analogs	Inhibition of nucleic acid synthesis	
Cancer	Ribonucleotide reductase inhibitors	Inhibit reduction of ribonucleotides (e.g. CTP) to deoxyribonuc-leotides (dCTP)	Hydroxyurea

Cancer	Nucleotide/nucleoside uptake inhibitors	Block import of cytotoxic pyrimi-dine analogs (protective effect), or block import of normal pyrimidine nucleotides, thereby reducing salvage synthesis and increasing need for de novo synthesis, including dTMP synthesis.	dipyridamole, BIBW 22 (a dipyridamole analog), nitroben-zylthioinosine

Table 9. Genes affecting the action of drugs which modulate pyrimidine metabolism. A variety of proliferative diseases, especially cancer, are treated with drugs that affect pyrimidine metabolism. All of the listed drugs are frequently used in combination with other drugs.

Other Pyrimidine Analogs

There are a large number of pyrimidine analogs in clinical development for a wide variety of indications. One of the most common indications is cancer and leukemia and lymphoma of various types. For example, 2',2'-difluorodeoxycytidine (gemcitabine; Gemzar) is a pyrimidine nucleoside drug with clinical efficacy in several common solid cancers; cytosine arabinoside (ARA-C) is another pyrimidine analog used in the treatment of leukemia; 2-chlorodeoxyadenosine and fludarabine (F-araA) are also used as antineoplastic drugs. 2'-deoxy-2'-(fluoromethylene) cytidine (MDL 101,731, Kyowa Hakko Kogyo Co.), 2',2'-difluorodeoxycytidine, 5-aza-2'deoxycytidine (decitabine), 5-azacytidine, 5-azadeoxycytidine, and __ are under development as antineoplastic drugs.

CNS Drugs — Pyrimidine Pathway

The pyrimidine nucleoside, uridine, has been proposed as a potential supplement in the treatment of psychosis based on its ability to reduce haloperidol-induced dopamine release. Thus, coadministration of uridine with haloperidol might enhance the antipsychotic action of standard neuroleptics, allowing for a reduction in dose and thereby a reduction in the frequency of side effects. The presumed mechanism is interaction with dopamine or GABA neurotransmission. The levels or function of pyrimidine transporters or pyrimidine *de novo* or salvage biosynthetic enzymes, or pyrimidine catabolic enzymes may affect the action of neuroleptics, or their modulation by pyrimidine nucleosides or pyrimidine analogs.

Other Therapeutics Relevant to the Pyrimidine Pathway

Another possible mode of pyrimidine nucleotide action is via stimulation of thromboxane A2 release from cultured glial cells. Uridine triphosphate, uridine diphosphate, cytidine triphosphate, and deoxythymidine triphosphate all induce concentration-dependent increases in the release of thromboxane A2 from cultured glial cells, indicating a possible role in brain response to damage *in vivo*.

Other cancers such as head and neck, breast, pancreas, other gastrointestinal cancers including stomach and intestinal may be directly targeted by therapeutic intervention that affects DNA methylation levels, pyrimidine synthesis, transport, and degradation pathways.

5   Many neurological diseases in both the CNS and the periphery may also be affected by therapeutic intervention of DNA methylation, pyrimidine synthesis, transport, and degradation pathways. Such intervention may be of therapeutic benefit to halt, retard, and or reduce symptoms of these often debilitating diseases.

10   **Example 9 – Drugs That Affect the Folate and Pyrimidine Pathways**

There are many potential candidate therapeutic interventions or drugs that can affect the folate and pyrimidine pathways. Categories of these are 5-FU prodrugs, drugs that affect DNA methylation pathways, and other drugs that have been developed for similar indications as 5-FU.

15

5-FU prodrugs

The clinical development and use of 5-FU prodrugs is further subject to improvement by the methods of this invention. These drugs are generally modified fluoropyrimidines that require one or more enzymatic activation steps for conversion 20 into 5-FU. The activation steps may result in prolonged drug half-life and/or selective drug activation (i.e. conversion to 5-FU) in tumor cells.

Examples of such drugs include capecitabine (Xeloda, Roche), a drug that is converted to 5-FU by a three-step pathway involving Carboxylesterase 1, Cytidine Deaminase and Thymidine Phosphorylase. Another 5-FU prodrug is 5'deoxy 5-FU 25 (Furtulon, Roche) which is converted to 5-FU by Thymidine Phosphorylase and/or Uridine Phosphorylase. Another 5-FU prodrug is 1-(tetrahydro-2-furanyl)-5-fluorouracil (FT, ftorafur, Tegafur, Taiho - Bristol Myers Squibb), a prodrug that is converted to 5-FU by cytochrome P450 enzyme, CYP3A4.

30   *Drugs acting on DNA methyation pathways*
        Antivirals

Herpes virus thymidine kinase phosphorylates many 5-substituted 2'-deoxyuridines, analogs of thymidine (e.g., idoxuridine, trifluridine, edoxudine, brivudine) and 5-substituted arabinofuranosyluracil derivatives (e.g., 5-Et-Ara-U, 35 BV-Ara-U, Cl-Ara-U). The 5'-monophosphates are further phosphorylated by cellular enzymes to the 5'-triphosphates, which are usually competitive inhibitors of the viral-coded DNA polymerases.

Unlike herpes viruses, retroviruses including but not limited to human immunodeficiency viruses do not encode specific enzymes required for the metabolism of the purine or pyrimidine nucleotides to their corresponding 5'-triphosphates. Therefore, 2',3'-dideoxynucleosides and acyclic nucleoside

5   phosphonates must be phosphorylated and metabolized by host cell kinases and other enzymes of purine and/or pyrimidine metabolism. In this way, affecting the pyrimidine synthetic, transport, or degradation pathways by candidate therapeutic intervention may be therapeutic beneficial in treating retroviral infections. Excamples of candidate antivirals that may be affected by alteration of pyrimidine

10   synthetic, transport, or degradation pathwyas are azidothymidine (AZT), acyclovir, and ganciclovir. These and other drugs have been used both as antivirals and antineoplastic agents.

Other Drugs Developed for Similar Indications as 5-FU

15   A variety of drugs are being developed for similar indications as 5-FU, and/or are being tested in combinations with 5-FU/leukovorin. These include the new platinum compound oxaliplatin (L-OHP) and the topoisomerase I inhibitors irinotecan (CPT11, Pharmacia-UpJohn) and topotecan. The effective clinical development or clinical use of these drugs may be enhanced by the methods of this

20   invention. In particular, identification of patients likely to respond to 5-FU with or withour leukovorin, may be useful in selecting optimal responders to other drugs. Alternatively identification of patients likely to suffer toxic response to 5-FU containing regimens may allow identification of patients best treated with other drugs. Other drugs with activity against cancers usually treated with regimens

25   containing 5-FU (e.g. metastatic colon cancer) include Suramin, a bis-hexasulfonated napthylurea; 6-hydroxymethylacylfulvene (HMAF; MGI 114); LY295501; bizelesin (U-7779; NSC615291), ONYX-015, monoclonal antibodies (e.g. 17-1A and MN-14), protein synthesis inhibitors such as RA 700, and angiogenesis inhibitors such as PF 4. Still other drugs may prevent colorectal cancer

30   by preventing the formation of colorectal polyps (eg, cyclooxygenase inhibitors may induce apoptosis of polyps).

Example 10

35   **Protocol for Clinical Trial for Determining the Relationship Between Toxicity of a Drug and Genetic Variances in Genes Related to the Action of the Drug**
     THIS EXAMPLE PROVIDES AN EXEMPLARY CLINICAL TRIAL AS A CASE CONTROL STUDY WHICH INCLUDES EVALUATING THE EFFECTS

OF SEQUENCE VARIANCES IN ENZYMES WHICH CAN MEDIATE THE
EFFECTS OF A KNOWN DRUG, IN THIS CASE IN AN ANTICANCER
TREATMENT. THE INFORMATION IN THE BACKGROUND SECTION OF
THIS PROTOCOL IS ALSO PROVIDED IN LARGE PART IN THE DETATILED
5   DESCRIPTION, BUT IS REPEATED HERE FOR COMPLETENESS OF THE
PROTOCOL DESCRIPTION.

PROTOCOL TITLE: Case-control study to determine the
relationship between toxicity of 5-fluorouracil (5-FU) given with folinic acid
10   (FA) to patients with solid tumors and DNA sequence variances in enzymes
that mediate the action of 5-FU and FA.

II.   *SIGNATURE PAGE*

5

10   _____

Name, position, and address of individual approving protocol from study sponsor.

15

_____

Name, position, and address of individual approving protocol from study sponsor.

20

III.   TABLE OF CONTENTS

IV. ACRONYMS AND ABBREVIATIONS

5-FU 5-Fluorouracil

FA   Folinic acid

5   °C   Degree centigrade

CBC   Complete blood count

CRF   Case report form

DCC   Data Coordinating Center

DMC   Data Monitoring Committee

10   EC   Ethical Committee

ECG   Electrocardiogram

e.g. For example

°F   Degrees Fahrenheit

FDA   Food and Drug Administration

15   i.e.   That is

IRB   Institutional Review Board

IV   Intravenous

mcg   Microgram

mg   Milligram

20   mL   Milliliter

mm^3 Cubic millimeter

PD   Pharmacodynamic

PK   Pharmacokinetic

®   Registered trade mark

25   REB   Research Ethics Board

USA   United States of America

USP   United States Pharmacopoeia

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V.   STUDY FLOW CHART

	File Research	Medical Visit
Selection of patients from the file	X	
Informed Consent Form signed		X
Inclusion/Exclusion criteria checking		X
Chart reporting		X
Demographic reporting		X
Blood sampling		X

5

VI.   1.   SUMMARY

Protocol
          Title: Case-control study to determine the relationship
5   between toxicity of 5-fluorouracil (5-FU) given with folinic acid (FA) to
patients with solid tumors and DNA sequence variances in enzymes that
mediate the action of 5-FU and FA.

     <u>VII.   Study</u>
10   <u>VIII.   Phase: Phase IV</u>

Study
Design: Single-center, case-control study.

15   Study
          Objectives: The primary objective of this study is to compare the variance
frequency distribution in the dihydropyrimidine dehydrogenase (DPD) gene between
two groups of patients with solid tumors, treated by weekly or monthly regimen of
5-FU+FA and defined by level of toxicity (graded according to the NCI common
20   toxicity criteria) as:
          - Group 1: patients with high toxicity (grade III / IV on NCI criteria)
          - Group 2: patients with minimal toxicity (grade 0 / I / II on NCI criteria)

          The secondary objectives of the study are to determine the DPD gene haplotype
25   ***frequency distribution and the variance and/or haplotype frequency distributions in***
selected genes (other than DPD gene) between two groups of patients with solid
tumors, treated by weekly or monthly regimen of 5-FU+FA and defined by level of
toxicity. Analyses will be done globally, then by regimen (monthly vs. weekly) and by
type of toxicity (gastrointestinal vs. bone marrow).
30

Number of Subjects: Ninety (90) patients, 45 in each group, will be included.

Study Population: Patients treated with 5-FU+FA for solid tumors at the
35   Massachusetts General Hospital, Dana-Farber Cancer Institute and Brigham and
Women's Hospital.

StudyGroups: Patients will be divided into two groups depending on the degree of toxicity they experienced with treatment, if any:

- patients with high toxicity (grade III / IV on NCI criteria),
- patients with minimal toxicity (grade 0 / I / II on NCI criteria)

5   Visit Schedule: One visit to sign the informed consent form and to collect blood sample.

Evaluation Parameter: Frequency distribution of gene alleles and haplotypes.

IX. *2.* *INTRODUCTION*

X. 2.1 Background

XI. 2.1.1 Potential for Improved Effectiveness of 5-FU and 5-FU/FA

Introduction

Chemotherapy of cancer involves use of highly toxic drugs with narrow therapeutic indices. Although progress has been made in the chemotherapeutic treatment of selected malignancies, most adult solid cancers remain highly refractory to treatment. Nonetheless, chemotherapy is the standard of care for most disseminated solid cancers. Chemotherapy often results in a significant fraction of treated patients suffering unpleasant or life-threatening side effects while receiving little or no clinical benefit; other patients may suffer few side effects and/or have complete remission or even cure. Any test that could predict response to chemotherapy, even partially, would allow more selective use of toxic drugs, and could thereby significantly improve efficacy of oncologic drug use, with the potential to both reduce side effects and increase the fraction of responders. Chemotherapy is also expensive, not just because the drugs are often costly, but also because administering highly toxic drugs requires close monitoring by carefully trained personnel, and because hospitalization is often required for treatment of (or monitoring for) toxic drug reactions. Information that would allow patients to be divided into likely responder vs. non-responder (or likely side effect) groups, only the former to receive treatment, would therefore also have a significant impact on the economics of cancer drug use.

Predicting Response to Chemotherapy

Several methods for predicting response to chemotherapy in individual patients have been investigated over the years, ranging from the use of biochemical markers to testing drugs on a patients cultured tumor cells. None of these methods has proven sufficiently informative and practical to gain wide acceptance. However, there are some specific examples of tests useful for predicting toxicity. For example, a diagnostic test to predict side effects associated with the antineoplastic drugs 6-mercaptopurine, 6-thioguanine and azathioprine has begun to gain wide acceptance,

particularly among pediatric oncologists. Severe toxicity of thiopurine drugs is associated with deficiency of the enzyme thiopurine methyltransferase (TPMT). Currently most TPMT testing is done using an enzyme assay, however the TPMT gene has been cloned and mutations associated with low TPMT levels have been

5   identified; genetic testing is beginning to supplant enzyme assays because genetic tests are more easily standardized and economical.

While there are no good tests that predict positive chemotherapeutic response, there is demonstrated utility to measuring estrogen and progesterone receptor levels in

10   cancer tissue before selecting therapy directed at modulating hormonal state. Measuring genetic variation in proteins that mediate the effects of chemotherapy drugs is in some respects analogous to measuring ER and PR levels, which mediate the effects of hormones.

15   *Clinical Use and Effectiveness of 5-FU and 5-FU/FA*

5-FU is a pyrimidine analog in clinical use since 1957. 5-FU is used in the standard treatment of gastrointestinal, breast and head and neck cancers. Clinical trials have also shown responses in cancer of the bladder, ovary, cervix, prostate and pancreas.

20   The remainder of this discussion will concern colorectal cancer. 5-FU is used both in the adjuvant therapy of Dukes Stage B and C cancer and in the treatment of disseminated cancer. 5-FU alone produces partial remissions in 10 - 30% of advanced colorectal cancers, however only a few percent of patients have complete remissions. In the last 15 years a variety of biochemically motivated strategies for

25   modulating 5-FU activity have been tested. For example, 5-FU has been used in combination with PALA, a pyrimidine synthesis inhibitor, to deplete cellular pools of UTP and thereby enhance formation of FUTP; in combination with methotrexate, to inhibit purine anabolism, leading to increased PRPP levels and consequent increased conversion of 5-FU to its active nucleotide metabolites; and in

30   combination with folinic acid, which increases intracellular pools of reduced folate, driving formation of the ternary inhibitory complex formed by 5,10 methylenetetrahydrofolate, FdUMP and thymidylate synthase. Levamisole, interferon and alkylating agents have also been used in combination with 5-FU. 5-FU/Levamisole and 5-FU/FA are widely used in the adjuvant treatment of colon

35   cancer, while 5-FU/FA is the most commonly used regimen for advanced colorectal cancer. Several prospective randomized trials of 5-FU/FA vs. 5-FU alone in patients with advanced cancer have demonstrated up to two fold higher response rates to 5-

FU/FA, while three of the studies also showed increased survival. Two major
dosing regimens are used: 5-FU plus low dose FA given for five consecutive days
followed by a 23 day interval, or once weekly bolus IV 5-FU plus high dose FA.
The higher FA dose results in plasma FA concentrations of 1 to 10 uM, comparable
5   to those required for optimal 5-FU/FA synergy in tissue culture, however low dose
FA (20 mg/m² vs. 500 mg/m²) has produced comparable clinical benefit. Ongoing
clinical trials are designed to further test new drug combinations. In summary,
relatively few patients - in the single digits - live longer as a result of 5-FU/FA,
although significantly more have partial disease remission. The factors that
10   determine which patients respond or have side effects are not known.

Toxicity of 5-FU and Folinic Acid

5-FU toxicity has been well documented in randomized clinical trials. Patients
15   receiving 5-FU/FA are at even greater risk of toxic reactions and must be monitored
carefully during therapy. A variety of side effects have been observed, affecting the
gastrointestinal tract, bone marrow, heart and CNS. The most common toxic
reactions are nausea and anorexia, which can be followed by life threatening
mucositis, enteritis and diarrhea. Leukopenia is also a problem in some patients,
20   particularly with the weekly dosage regimen. In a recent randomized trial of weekly
vs. monthly 5-FU/FA there were 7 deaths related to drug toxicity among 372 treated
patients (1.9%; Buroker et al. 1994). 31% of patients receiving the weekly regimen
suffered diarrhea-requiring hospitalization for a median of 10 days. Other severe
toxicity, which occurred at lower frequency, included leukopenia and stomatitis. In
25   another example, 36% of patients receiving weekly bolus 5-FU plus FA (500
mg/m²), in a NSABP trial suffered NCI grade 3 toxicity (Wolmark et al., 1996).
Clearly, toxicity is a major cost of 5-FU/FA therapy, measured both in patient
suffering and in financial terms (the cost of care for drug induced illness).

30   *Other Factors*

Many non-genetic factors influence the response of cancers to drugs, including
tumor location, vasculature, cell growth fraction and various drug resistance
mechanisms. It will therefore not be possible to explain all heterogeneity in
35   response to 5-FU/FA by genetic variation. However, based on genetic studies of
other quantitative traits it seems likely that a significant fraction of variation in drug
response can be explained (see below).

XII. 2.1.2 Metabolic Pathways that Affect 5-FU/FA Action

The biochemical pathways of 5-FU metabolism have been studied extensively.
5   Likewise, folate metabolism has been well investigated and the enzymes that form
and consume 5, 10-methylenetetrahydrofolate are well known. The principal
metabolic pathways that influence the pharmacologic action of 5-FU are
summarized in Figure 1.

10   **Figure 1. 5-FU metabolism and inhibition of thymidylate formation.** Enzymes:
1. uridine phosphorylase; 2. thymidine phosphorylase; 3. orotate phosphoribosyl
transferase; 4. thymidine kinase; 5. uridine kinase; 6. ribonucleotide reductase; 7.
thymidylate synthase; 8. dCMP deaminase; 9. nucleoside monophosphate kinase;
10. nucleoside diphosphate kinase; 11. nucleoside diphosphatase or cytidylate
15   kinase; 12: thymine phosphorylase. FH2 = dihydrofolate, FH4 = tetrahydrofolate.
The Figure is adapted from Goodman & Gilman's The Pharmacological Basis of
Therapeutics, ninth edition, McGraw Hill, 1996, p. 1249.

De novo and salvage routes of pyrimidine nucleotide formation (5-FU anabolism)
20   *and inhibition of thymidylate synthase*

5-FU is a biologically inactive pyrimidine analog, which must be phosphorylated,
and ribosylated to the nucleoside analog fluorodeoxyuridine monophosphate
(FdUMP) to have clinical activity. FdUMP formation can occur via several routes,
25   summarized in Figure 1. 5-FU may be converted by uridine phosphorylase to
fluorouridine (FUdR; the reverse reaction is catalyzed by uridine nucleosidase) and
then to fluorouridine monophosphate (FUMP) by uridine kinase, or FUMP may be
formed from 5-FU in one step via transfer of a phosphoribosyl group from 5-
phosphoribosyl-1-pyrophosphate (PRPP), catalyzed by orotate phosphoribosyl
30   transferase. FUMP can be converted to FUDP and subsequently FUTP by a
nucleoside monophosphate kinase and nucleoside diphosphate kinase, respectively.
FUTP is incorporated into RNA by RNA polymerases, which may account in part
for 5-FU toxicity as a result of effects on processing or function (e.g. translation).
Alternatively, FUDP may be reduced to the dinucleotide level, FdUDP
35   (fluorodeoxyuridine diphosphate) by ribonucleotide diphosphate reductase, a
heterodimeric enzyme. FdUDP can then be converted to FdUTP by nucleoside
diphosphate kinase and incorporated into DNA by DNA polymerases, which may

account for some 5-FU toxicity. Fluoropyrimidine modified DNA may also be
targeted by the nucleotide excision repair process. The more important path of
FdUDP metabolism with respect to anticancer effects, however, is believed to be
conversion to FdUMP by nucleoside diphosphatase (or cytidylate kinase, a bi-
directional enzyme). dUMP is the precursor of dTMP in de novo pyrimidine
biosynthesis, a reaction catalyzed by thymidylate synthase and which consumes
5,10-methylenetetrahydrofolate, producing 7,8 dihydrofolate. FdUMP, however,
forms an inhibitory (probably covalent) complex with thymidylate synthase in the
presence of 5,10-methylenetetrahydrofolate, thereby blocking formation of
thymidylate (other than by the salvage pathway via thymidine kinase). The complex
anabolism of FdUMP can be simplified by giving the deoxyribonucleoside of 5-FU,
5-fluorodeoxyuridine (also called floxuridine; FUdR), which can be converted to
FdUMP in one step by thymidine kinase. However, FUdR is also rapidly converted
back to 5-FU by the bi-directional enzyme thymidine phosphorylase.

5-FU catabolism.

Metabolic elimination of 5-FU occurs via a three-step pathway leading to -alanine.
The first and rate limiting enzyme in the elimination pathway is dihydropyrimidine
dehydrogenase (DPD), which transforms more than 80% of a dose of 5-FU to the
inactive dihydrofluorouracil form. Subsequently dihydropyrimidinase catalyzes
opening of the pyrimidine ring to form 5-fluoro--ureidopropionate and then -
ureidopropionase (also called -alanine synthase) catalyzes formation of 2-fluoro--
alanine. The first two reactions are reversible. The distribution of activity of these
enzymes in human populations has not been established, however, a recent
population survey of urinary pyrimidine levels in 1,133 adults revealed that levels of
dihydrouracil range from 0 - 59 uM/g of creatinine, while uracil levels ranged from 0
- 130 uM/g creatinine (Hayashi et al., 1996), suggesting variation in the activity of
enzymes of pyrimidine metabolism. It is worth noting that in animal studies
catabolites of 5-FU apparently account for some fraction of 5-FU toxicity (Davis et
al., 1994; Spector et al., 1995). This result is the rationale for current human trials of
5-FU combined with DPD inhibitors: if the 5-fluoro- metabolites are responsible for
toxicity, then blocking their formation by inhibition of DPD, while simultaneously
decreasing 5-FU dosage to compensate for the block in catabolism and excretion,
should result in a better therapeutic index.

Folinic acid conversion to tetrahydrofolate.

The conversion of FA to 5,10MTHF can occur via several routes, illustrated in
Figure 2

5   **Figure 2. Folate metabolism and formation of 5,10-methylenetetrahydrofolate.**
Enzymes: 1. Formimino-tetrahydrofolate cyclodeaminase; 2.
methenyltetrahydrofolate synthetase; 3. methenyltetrahydrofolate cyclohydrolase; 4.
formyltetrahydrofolate synthetase; 5. formyltetrahydrofolate hydrolase; 6.
formyltetrahydrofolate dehydrogenase; 7. methylenetetrahydrofolate dehydrogenase;
10   8. methylenetetrahydrofolate reductase (MTHFR); 9. homocysteine
methyltransferase (also called methionine synthetase); 10. serine
transhydroxymethylase; 11. glycine cleavage system; 12. thymidylate synthase; 13.
dihydrofolate reductase. Abbreviations: THF = tetrahydrofolate; DHF =
dihydrofolate. Note that THF appears twice (i.e. the product of step 6 is also
15   substrate for enzymes 10 and 11. Step 12 also appears in Figure 1, above. This
Figure is adapted from Mathews & van Holde, Biochemistry, The
Benjamin/Cummings Publishing Co., Redwood City CA, 1990, page 697.

Intracellular reduced folate levels can potentiate 5-FU action by increasing 5,10-
20   methyl-enetetrahydrofolate levels (5,10-methyleneTHF; see center of Figure 2),
thereby stabilizing the ternary inhibitory complex formed with thymidylate synthase
and FdUMP. This is the basis for therapeutic modulation of 5-FU with FA. As can
be seen in Figure 2, conversion of folinic acid (5-formylTHF) to 5,10-methenylTHF,
the precursor of 5,10-methyleneTHF, requires methenyltetrahydrofolate synthetase
25   (enzyme 2 in the Figure). Also, levels of 5,10-methyleneTHF may be affected
directly by the activity of methylenetetrahydrofolate dehydrogenase,
methylenetetrahydrofolate reductase, serine transhydroxymethylase and the glycine
cleavage system enzymes (7, 8, 10 and 11 in Fig. 2), and indirectly by the other
enzymes shown in the Figure.
30

Cell uptake of pyrimidine nucleosides and folinic acid

Human cells have five concentrative nucleoside transporters with varying patterns of
tissue distribution (see review by Wang et al., 1997). Two transporters, one with
35   preference for purines and one for pyrimidines have been cloned recently (Felipe et
al., 1998). 5-FU entry into cells may be modulated by activity of these transporters,
particularly the pyrimidine transporter, although one prospective randomized clinical

trial in which the nucleoside transport inhibitor dipyridamole was paired with 5-FU and FA failed to show a difference in outcome compared to 5-FU/FA alone (Kohne et al., 1995). Several folate transport systems have been identified in human cells. Folate receptor 1 (FR1) is a high affinity (nanomolar range) receptor for reduced

5   folates. Three restriction fragment length polymorphisms (RFLPs) have been reported at the FR1 locus (Campbell et al., 1991). Reduced folates are also transported by folate receptor gamma and by a low affinity (1 uM) folate transporter. 15-fold variations in levels of folate transporter have been described in unselected tumor cell lines (Moscow et al., 1997).

10

XIII.   2.1.3 Genetically Determined Variation in Response to 5-FU: Studies of Dihydropyrimidine Dehydrogenase Deficiency

Dihydropyrimidine Dehydrogenase Deficiency is Associated with 5-FU Toxicity

15

5-FU is inactivated by the same metabolic pathway as thymine and uracil (see above). DPD catalyzes the first, rate-limiting step in pyrimidine catabolism and accounts for elimination of most 5-FU. Normal individuals eliminate 5-FU with a half-life of ~10-15 minutes and excrete only 10% of a dose unchanged in the urine.

20   In contrast, people genetically deficient in DPD eliminate 5-FU with a half-life of ~2.5 hours and excrete 90% of a dose unchanged in the urine (Diasio et al., 1988). DPD deficiency has two clinical presentations: (i) an inborn error of metabolism causing some degree of neurologic dysfunction or (ii) asymptomatic until revealed by exposure to 5-FU or other pyrimidine analogs. With either presentation there is

25   combined hyperuraciluria and hyperthyminuria. The vastly increased 5-FU half-life in DPD deficient individuals causes severe toxicity and even death. Recently several mutations have been identified in DPD genes of deficient individuals (Wei et al., 1996), however none of these alleles appears to occur at appreciable frequency, so the cause of wide population variation in DPD levels is still not understood.

30

Population Studies of DPD Activity Show Wide Variation

Population surveys of DPD activity in normal individuals have been performed using blood and liver samples. These studies reveal a broad unimodal Gaussian

35   distribution of DPD activity over a 7 to 14 fold range, with some individuals having very low or even undetectable levels. For example Etienne et al. (1994) report DPD activity ranging from .065 to .559 nM/min/mg protein in a study of 152 men and 33

women, while Fleming et al. (1993) found DPD activity in 66 cancer patients varied from .17 to .77 nM/min/mg protein. Lu et al (1995) found 18-fold variation in liver DPD assayed in 138 individuals. Milano and Etienne (1994) suggested that the frequency of heterozygous and homozygous deficiency is 3% and .1%, respectively.

5 The DNA sequence alterations responsible for null DPD alleles do not account for the high population variability (Ridge et al., 1997).

DPD Levels are correlated with Response to 5-FU

10 Intratumoral DPD levels have been measured in patients receiving 5-FU chemotherapy. When complete responders were compared to partial or non-responders, DPD levels were lower in the compete responders (Etienne et al., 1995). Leukocyte DPD levels has also been measured in patients receiving 5-FU/FA chemotherapy. When patients were divided into 3 groups: high, medium and low

15 DPD activity, the frequency of serious side effects was highest in the low DPD group and vice versa (Katona et al., 1997).

XIV. 2.1.4 Variances in Genes That May Affect 5-FU/FA Action

20 Variagenics has already surveyed thymidylate synthase, ribonucleotide reductase (M1 subunit only), and dihydrofolate reductase and dihydropyrimidine dehydrogenase cDNAs for genetic variation. 36 unrelated individuals were screened using 6 SSCP conditions and DNA sequencing. Other investigators have identified variances in MTHFR, methionine synthase and folate receptor. These findings are

25 summarized in Appendix I.

XV.

XVI. 2.1.5 Analysis of Haplotypes Increases Power of Genetic Analysis

It is evident from work to date that, while DPD activity is weakly predictive of 5-FU

30 toxicity and drug response, there must be other factors that account for some of the variation in patient response. This is to be expected as drug response phenotypes usually vary continuously, and such (quantitative) traits are typically influenced by a number of genes (Falconer and Mackay, 1997). Although it is impossible to determine *a priori* the number of genes influencing a quantitative trait, often only a

35 few loci have large effects, where a large effect is 5-20% of total variation in the phenotype (Mackay, 1995).

Having identified genetic variation in enzymes that may affect 5-FU action, how can we most efficiently address its relation to phenotypic variation? The sequential testing for correlation between phenotypes of interest and single nucleotide polymorphisms may be adequate to detect associations if there are major effects

5   associated with single nucleotide changes; certainly it is worth performing this type of analysis. However there is no way to know in advance whether there are major phenotypic effects associated with single nucleotide changes and, even if there are, there is no way to be sure that the salient variance has been identified by screening cDNAs. A more powerful way to address the question of genotype-phenotype

10   correlation is to assort genotypes into haplotypes. (A haplotype is the cis arrangement of polymorphic nucleotides on a particular chromosome.) Haplotype analysis has several advantages compared to the serial analysis of individual polymorphisms at a locus with multiple polymorphic sites.

15   (1)   Of all the possible haplotypes at a locus (2^n haplotypes are theoretically possible at a locus with n binary polymorphic sites) only a small fraction will generally occur at a significant frequency in human populations. Thus, association studies of haplotypes and phenotypes will involve testing fewer hypotheses. As a result there is a smaller probability of Type I errors, that is, false inferences that a

20   particular variant is associated with a given phenotype.

(2)   The biological effect of each variance at a locus may be different both in magnitude and direction. For example, a polymorphism in the 5' UTR may affect translational efficiency, a coding sequence polymorphism may affect protein

25   activity, a polymorphism in the 3' UTR may affect mRNA folding and half life, and so on. Further, there may be interactions between variances: two neighboring polymorphic amino acids in the same domain - say cys/arg at residue 29 and met/val at residue 166 - may, when combined in one sequence, for example, 29cys-166val, have a deleterious effect, whereas 29cys-166met, 29arg-166met and 29arg-166val

30   proteins may be nearly equal in activity. Haplotype analysis is the best method for assessing the interaction of variances at a locus.

(3)   Templeton and colleagues have developed powerful methods for assorting haplotypes and analyzing haplotype/phenotype associations (Templeton et al.,

35   1987). Alleles, which share common ancestry, are arranged into a tree structure (cladogram) according to their time of origin in a population. Haplotypes that are evolutionarily ancient will be at the center of the branching structure and new ones

(reflecting recent mutations) will be represented at the periphery, with the links representing intermediate steps in evolution. The cladogram defines which haplotype-phenotype association tests should be performed to most efficiently exploit the available degrees of freedom, focusing attention on those comparisons

5   most likely to define functionally different haplotypes (Haviland et al., 1995). This type of analysis has been used to define interactions between heart disease and the apolipoprotein gene cluster (Haviland et al 1995) and Alzheimer's Disease and the Apo-E locus (Templeton 1995) among other studies, using populations as small as 50 to 100 individuals.

10

XVII. 2.1.6 Biochemical Studies of Alternate Allelic Forms of DPD

The power of genetic analysis can be augmented by biochemical studies of alternate allelic forms of enzymes. Biochemical data on the distribution of activity of a series

15 of enzymes in a biochemical pathway provides the basis for metabolic flux analysis (Keightly, 1996). It is beyond the scope of this clinical trial to analyze biochemical variation in the enzymes of pyrimidine and folate metabolism. However, since Variagenics has identified new variances in DPD that may plausibly affect enzyme expression or activity, and because DPD is already proven to play a role in 5-FU

20 response, parallel studies will be conducted to investigate the relationship between genotype and biochemistry for this enzyme.

DPD cDNAs have been cloned from a variety of higher eukaryotes and binding sites for its cofactors, prosthetic groups and substrate have been defined experimentally or

25 by analogy with known consensus motifs (Yokata et al., 1994). The DPD polymorphisms that affect protein sequence occur at amino acids 29 (cys/arg) and 166 (met/val) in the amino-terminal one-third of the protein. Phylogenetic comparison of this region from boar, human, cow, fly, and bacteria (see below) shows that there are actually two highly conserved motifs that resemble either

30 iron/sulfur or zinc binding motifs, the latter being more likely due to the spacing of the cysteine residues. The region around the met/val polymorphism at amino acid 166 is highly conserved. Even the spacing of the putative zinc-finger domains is maintained between distantly related species, hinting at their importance. Since amino acid 166 is close to a highly conserved (and probably functionally important)

35 region and is itself conserved, being a methionine in all species, it seems likely that perturbations in this position would have consequence. The polymorphism substitutes a long amino acid side chain capable of hydrogen bonding (methionine)

for a compact, hydrophobic amino acid (valine). The region around amino acid 29 is not as well conserved.

XVIII. 2.2   Study Rationale

5

5-fluorouracil (5-FU) is a fluorinated pyrimidine analog that is widely used in chemotherapy. The effectiveness of 5-FU is potentiated by folinic acid (FA: generic name: leukovorin). The combination of 5-FU and FA is standard therapy for stage III/IV colon cancer. Patient responses to 5-FU and 5-FU/FA vary widely, ranging

10   from complete remission of cancer to severe toxicity.

Pyrimidine base analogs are degraded by the same enzymes that degrade endogenous uracil and thymine. Dihydropyrimidine dehydrogenase (DPD) is the first degradative enzyme in this pathway, accounting for catabolism of more than

15   80% of an administered dose of 5-FU.

Total DPD deficiency (familial pyrimidinemia and pyridinuria) is a rare syndrome associated with 5-FU induced toxicity. A milder defect in DPD activity appears to account for the severe side effects that occur in 1%-3% of unselected cancer patients

20   (Milano and Etienne, 1994).

The major toxic manifestations of 5-FU and FA depend on the schedule of administration and occur mainly in rapidly dividing tissues such as bone marrow and the mucosal lining of the gastrointestinal tract.

25

This study is designed to test whether genetically encoded biochemical variations in the enzymes of pyrimidine catabolism, nucleotide metabolism and folic acid metabolism, among patients treated with a weekly or monthly schedule of 5-FU+FA, account for some of the variation in drug toxicity. Applications of a successful

30   pharmacogenetic study lie in the direction of safer, more efficacious, and hence more economical use of 5-FU, guided by genetic tests.

XIX.   3.   OBJECTIVES

35

XX.   3.1   Primary Objective

The primary objective of this study is to compare the variance frequency distribution in the dihydropyrimidine dehydrogenase (DPD) gene between two groups of patients with solid tumors, treated by weekly or monthly regimen of 5-FU+FA and defined by level of toxicity (graded according to the NCI common

5   toxicity criteria) as:

- Group 1: patients with high toxicity (grade III / IV on NCI criteria)
- Group 2: patients with minimal toxicity (grade 0 / I / II on NCI criteria)

XXI.   3.2   Secondary Objectives

10

The secondary objectives of the study are to determine the DPD gene haplotype frequency distribution and the variance and/or haplotype frequency distributions in selected genes (other than DPD gene –see Appendix I-) between two groups of patients with solid tumors, treated by weekly or monthly regimen of 5-FU+FA and defined by

15   level of toxicity. Analyses will be done globally, then by regimen (monthly vs. weekly) and by type of toxicity (gastrointestinal vs. bone marrow).

20   *XXII.   4.   STUDY DESIGN*

XXIII.4.1   Study Outline

The study will be done at *selected medical institution.*

25

The study is a single-center, case-control study. The duration of the study is expected to be not more than 8 months.

Genetic analysis of anonymized patient samples will take place at the *study sponsor.*

30

XXIV. 4.2   Subject Withdrawal from the Study

Subjects who desire to discontinue participation in this study must be withdrawn from the study.

35

XXV.   4.3   Discontinuation of the Study

This study may be terminated by *the study sponsor*, after consultation with the Advisory Committee (see Section 11.2), at any time.

5 *XXVI.* *5.* *STUDY POPULATION*

XXVII.   5.1   Number of Subjects

Ninety (90) subjects will be recruited for the study.

10

XXVIII.   5.2   Inclusion Criteria

To be eligible for entry into this study, candidates must meet the following eligibility criteria at the time of enrollment:

15

1. Above age of 18 years.

2. Diagnosis of solid tumor.

20   3. Treatment with a weekly or monthly regimen of 5-fluorouracil (5-FU) plus folinic acid (FA)

4. Classified according to the NCI common toxicity criteria as 0, I, II, III or IV grade.

25

5. Give written informed consent prior to any testing under this protocol, including screening tests and evaluations that are not considered part of the subject's routine care.

30   ### XXIX. 5.3   Exclusion Criteria

Candidates will be excluded from study entry if any of the following exclusion criteria exist at the time of enrollment:

35   *Medical History*

1. Diagnosis of cancer other than solid tumor.

2. Classified according to the NCI common toxicity criteria as grade II.

3. Known history of HIV, HBV or Hepatitis C virus infection (undesirable for making permanent cell line).

Treatment History

4. Treatment with 5-FU + FA but with other schedule than weekly or monthly.

5. Concomitant treatment with other cancer drugs than 5-FU+FA.

Miscellaneous

6. Unwillingness or inability to comply with the requirements of this protocol.

XXX. 5.4 Screening Log

For every patient initially considered for inclusion in this study, it is required to document and to specifically state the reason(s) for their exclusion.

XXXI. 6. ALLOCATION PROCEDURE

When the eligibility review screening has been completed and the subject has been found eligible for admission to the study, the subject will be assigned to one of the two following group, depending on the 5-FU+FA related toxicity he has experienced in the past:

- Group 1: patients with high toxicity (grade III / IV on NCI criteria)
- Group 2: patients with minimal toxicity (grade 0 / I / II on NCI criteria)

7. SCHEDULE OF EVENTS

XXXII. Patients

Patients will only be required to come for giving informed consent, then having one blood drawing (17ml total) –see Appendix II-.

5   *Study Personnel*
The following personnel will be involved in the conduct of this study.

   • A *treating physician* who will oversee subject assignment and discuss the protocol with the subject in order to obtain informed consent.

10   • A *treating nurse* who will assist the treating physician in subject identification management and perform blood sampling.

   • A *data manager* who will collect and enter data in the clinical database.

15   *Tests and Evaluations*
The tests and evaluations described below must be performed *by the required study personnel* in order to determine subject eligibility.

Treating physician

20   • Chart and demographic (sex, age, etc) reporting, inclusion/exclusion criteria checking.

Treating nurse
   • Blood sampling

25

Data manager
   • Clinical data entry.

30   *XXXIII.   11.   STATISTICAL STATEMENT AND ANALYTICAL PLAN*

XXXIV.   11.1   Sample Size Considerations

35   The primary endpoint of this study is to measure and compare genotype distributions of the DPD gene in patients with and without 5-FU+FA toxicity. In order to be able to make a sample size calculation, we

will ignore the complexities of the underlying genetic model and treat the data as n independent ordinary 2 x 2 contingency tables for the n variances in the cases and controls. So, using the 2 most frequent DPD variances listed in Appendix 1 and an odds-ratio of 4.00 for cases vs. controls, we can determine the sample size for every variance, with an equal number of subjects in each phenotypic (i.e. toxicity) group, required to detect, with 80% power at a two-sided significance level of 0.05, a statistically significant difference between distributions:

           - nucleotide 3925:   44 patients per group

           - nucleotide 3937:   43 patients per group.

A total of 90 patients (45 per group) will so be recruited.

11.2   Description of Objectives and EndpointS

XXXV.   **11.2.1 Primary Objective and Endpoints**

The primary objective of this study is to compare the variance frequency distributions in the dihydropyrimidine dehydrogenase (DPD) gene between two groups of patients with solid tumors, treated by weekly or monthly regimen of 5-FU+FA and defined by level of toxicity (grade 0/I/II vs. grade III/IV).

XXXVI.   **11.2.2 Secondary Objectives and Endpoints**

The secondary objectives of the study are:

1.   To determine which DPD gene variance(s) is(are) associated to 5-FU+FA toxicity

2.   To determine which DPD haplotype(s) is(are) associated to 5-FU+FA toxicity.

3.   To determine if one or more of the other gene variances (see Appendix 1) is(are) associated to 5-FU+FA toxicity

4.   To determine if one or more of the other haplotypes is(are) associated to 5-FU+FA toxicity.

11.3   CRiteria for the Endpoints

5   Since we do not know the mode of inheritance of a potential toxic susceptibility, we will ignore in a first step the complexities of the underlying genetic model and treat the data as an ordinary $n \times 2$ contingency table for the n variances in the cases and controls.

Then, for every variance, we will compare genotype frequencies in order to detect a

10   potential effect of homo- vs. heterozygosity.

We will also compare haplotype frequencies of r predetermined haplotypes. The method of cladograms (Templeton et al., 1987) will be used in an attempt to find out the smallest possible number r. In this method the evolutionary relationships between present day haplotypes are represented as a tree or cladogram.

15

XXXVII.   11.4   Statistical Methods To Be Used in Objective Analyses

The statistical significance of the difference between variance frequencies will be

20   assessed by a Pearson chi-squared test of homogeneity of proportions with $n-1$ degrees of freedom. Then, in order to determine which variance(s) is(are) responsible for an eventual significance, we will consider each variance individually against the rest, yielding up to n comparisons each based on a 2 x 2 table. This should result in chi-squared tests that are individually valid but taking the most

25   significant of these tests is a form of multiple testing. A Bonferroni's adjustment for multiple testing will so be made to the P-values such as $p^* = 1-(1-p)^n$.

The statistical significance of the difference between genotype frequencies associated to every variance will be assessed by a Pearson chi-squared test of

30   homogeneity of proportions with 2 degrees of freedom, using the same Bonferroni's adjustment as above.

Testing for unequal haplotype frequencies between cases and controls can be considered in the same framework as testing for unequal variance frequencies since a

35   single variance can be considered as a haplotype of a single locus. The relevant likelihood ratio test compares a model where two separate sets of haplotype frequencies apply to the cases and controls, to one where the entire sample is

characterized by a single common set of haplotype frequencies. This can be performed by repeated use of a computer program (Terwilliger and Ott, 1994) to successively obtain the log-likelihood corresponding to the set of haplotype frequency estimates on the cases ($\ln L_{case}$), on the controls ($\ln L_{control}$) and on the

5   overall ($\ln L_{combined}$). The test statistic $2(\ln L_{case} + \ln L_{control} - \ln L_{combined})$ is then a chi-squared with $r-1$ degrees of freedom (where r is the number of haplotypes).

To test for potential confounding effects or effect-modifiers, such as sex, age, etc. logistic regression will be used with case-control status as the outcome variable, and

10   genotypes and covariates (plus possible interactions) as predictor variables.

XXXVIII.   *12.*   *ETHICAL REQUIREMENTS*

15   **XXXIX.   12.1   Declaration of Helsinki**

See Appendix III.

XL.   12.2   Subject Information and Consent

20

Prior to any testing under this protocol, including screening tests and evaluations, written informed consent must be obtained from the subject in accordance with the Standards of the Partners Cancercare Human Protection Committee (HPC).

25   The background of the proposed study and the benefits and risks of the procedures and study will be explained to the subject. A copy of the informed consent document signed and dated by the subject must be given to the subject. Confirmation of a subject's informed consent must also be documented in the subject's medical records prior to any testing under this protocol, including

30   screening tests and evaluations.

XLI.   12.3   Subject Data Protection

The subject will not be identified by name or other any identifying characteristic in

35   any study reports, and these reports will be used for research purposes only. *the study sponsor*, its designee(s), and various Government Health Agencies may inspect the

records of this study. All relevant demographic and historical data regarding patient drug response will be recorded in an anonymized database.

5 XLII. 13. *FURTHER REQUIREMENTS AND GENERAL INFORMATION*

XLIII. 13.1   Study Committee

10 *Advisory Committee*
An Advisory Committee will be formed to provide scientific and medical direction for the study and to oversee the administrative progress of the study. The Advisory Committee will meet at least once a month to monitor subjects. The Advisory Committee will determine whether the study should be stopped or amended for any
15 reason.

The Advisory Committee will be comprised of the Director of Clinical Pharmacogenetics, Vice-President for Discovery Research from *the study sponsor* (and/or their designee) and participating investigators. The principal investigator will
20 chair the Advisory Committee.

XLIV. 13.2   Changes to Final Study Protocol

All protocol amendments must be submitted to the IRB/REB/EC. Protocol
25 modifications that impact on subject safety, the scope of the investigation, or affect the scientific quality of the study must be approved by the IRB/REB/EC and submitted to the appropriate regulatory authorities before initiation. However, Variagenics may, at any time, amend this protocol to eliminate an apparent immediate hazard to a subject. In this case, the appropriate regulatory authorities
30 will be subsequently notified. In the event of a protocol modification, the subject consent form may require similar modifications.

XLV. 13.3   Record Retention

35

The Principal Investigator must maintain the records of signed consent forms, CRFs, all correspondences, dates of any monitoring visits, and records that support this

information for a period of 15 years following notification by *the study sponsor* that the clinical investigations have been completed or discontinued. All local laws regarding retention of records must also be followed.

XLVI. 13.4   Reporting and Communication of Results

All information concerning *the study sponsor's* perations, such as patent applications, formulas, manufacturing processes, basic scientific data, and formulation information supplied by *the study sponsor* and not published previously, are considered confidential and shall remain the sole property of the *study sponsor*. The investigator agrees to use this information only in conducting this study and shall not use it for any other purposes without *the study sponsor's* written approval. The investigator agrees not to disclose *the study sponsor's* confidential information to anyone except to people involved in the study who need such information to assist in conducting the study and then only on like terms of confidentiality and nonuse.

It is understood by the investigator that the information developed from this clinical study will be used by *the study sponsor* and therefore may be disclosed as required to other clinical investigators, to the U.S. Food and Drug Administration, the Canadian Health and Welfare Health Protection Branch, the European Medicines Evaluation Agency, and to other government agencies. In order to allow for the use of the information derived from the clinical studies, it is understood that there is an obligation to provide *the study sponsor* with complete test results and all data developed in the study.

No publication or disclosure of study results will be permitted except as specified in a separate, written agreement between *the study sponsor* and the investigator.

XLVII.   13.5   PROTOCOL COMPLETION

The IRB/REB/EC must be notified of completion or termination of the protocol. Within 3 months of protocol completion or termination, the investigator must provide a final clinical summary report to the IRB/REB/EC. The Principal Investigator must maintain an accurate and complete record of all submissions made to the IRB/REB/EC, including a list of all reports and documents submitted. A copy of these reports should be sent to *the study sponsor*.

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XLIX. SIGNED AGREEMENT OF THE STUDY PROTOCOL

I have read the foregoing protocol, VRG-9801, "Case-control study to determine the relationship between toxicity of 5-fluorouracil (5-FU) given
5   with folinic acid (FA) to patients with solid tumors and DNA sequence variances in enzymes that mediate the action of 5-FU and FA", Version 1, and agree to conduct the study as detailed herein and to inform all who assist me in the conduct of this study of their responsibilities and obligations.

10

15   ⎯
       Principal Investigator's Signature   Date

       ⎯
20   Principal Investigator's Name (Print)

       ⎯
       Investigational Site (Print)

APPENDIX II

L.   Procedures for handling blood samples for cell line establishment

5   This document describes procedures for handling blood samples from cancer
patients enrolled in trial for genetic studies at *the study sponsor*. The approach will
be to first establish permanent lymphoblastoid cell lines. DNA and RNA will
subsequently be extracted from these cell lines. This procedure will save the effort
of purifying DNA and RNA directly from blood. Since the pharmacogenetic

10   hypotheses to be investigated relate to the effect of genotype, not mRNA expression
levels, lymphoblastoid cell lines should be satisfactory sources of nucleic acid for
the genetic studies.

1.   Cell line establishment will be done by the *study site institutions* (e.g.,
15   Genomics Core Facility of the Massachusetts General Hospital (MGH) Molecular
Neurogenetics Unit).

2.   From each patient collect two 8.5 ml yellow topped tubes (containing ACD
solution A) for lymphoblastoid cell line development. All DNA and RNA will be
20   produced from the cell lines at a later date; therefore there is no need for additional
· blood drawing.

3.   Fill out a DNA/Cell Line Order Sheet. An example is attached. Please note
that the patient's name should be anonymized at this point. (The Genomics Core
25   Facility will accept anonymized order forms.) All samples (including those for PK
studies) should be assigned the same arbitrary number to allow subsequent matching
of clinical, pharmacokinetic and genetic data. Also, the date and time of blood
drawing should be marker. DOB should be recorded as month and year only, and
sex should be recorded. Record the number of tubes of blood drawn (2), date of
30   draw and date of shipment. Under "Requisition" check off "Transformation only".

4.   Arrange for the two ACD blood samples to be delivered to *designated
individual at the study site institution* at the address given below:

35   *Name and address of designated individual at study site institution.*

Since the blood samples are typically aged at room temperature for a day or two before cell line establishment, it is not urgent that blood be delivered the same day it is drawn. Storage overnight, if necessary, should be at room temperature.

5.   Please fax to *the study sponsor* a copy of the cell line order form so we are aware of accumulating cell lines. The fax number is 588-5399. Please fax to the attention of *the designated individual for the study sponsor.*

6.   Once cell lines are established, vials will be archived at the *study site institution*, where they will be available to investigators.

7.   Questions for *the study sponsor* should be addressed to *the designated individual.*

Example 11 Example 10
Hardy-Weinberg equilibrium

Evolution is the process of change and diversification of organisms through time, and evolutionary change affects morphology, physiology and reproduction of organisms, including humans. These evolutionary changes are the result of changes in the underlying genetic or hereditary material. Evolutionary changes in a group of interbreeding individuals or Mendelian population, or simply populations, are described in terms of changes in the frequency of genotypes and their constituent alleles. Genotype frequencies for any given generation is the result of the mating among members (genotypes) of their previous generation. Thus, the expected proportion of genotypes from a random union of individuals in a given population is essential for describing the total genetic variation for a population of any species. For example, the expected number of genotypes that could form from the random union of two alleles, A and a, of a gene are AA, Aa and aa. The expected frequency of genotypes in a large, random mating population was discovered to remain constant from generation to generation; or achieve Hardy-Weinberg equilibrium, named after its discoverers. The expected genotypic frequencies of alleles A and a (AA, 2Aa, aa) are conventionally described in terms of $p^2 + 2pq + q^2$ in which p and q are the allele frequencies of A and a. In this equation ($p^2 + 2pq + q^2 = 1$), p is

defined as the frequency of one allele and q as the frequency of another allele for a trait controlled by a pair of alleles (A and a). In other words, p equals all of the alleles in individuals who are homozygous dominant (AA) and half of the alleles in individuals who are heterozygous (Aa) for this trait. In mathematical terms, this is

$$p = AA + \tfrac{1}{2}Aa$$

Likewise, q equals the other half of the alleles for the trait in the population, or

$$q = aa + \tfrac{1}{2}Aa$$

Because there are only two alleles in this case, the frequency of one plus the frequency of the other must equal 100%, which is to say

$$p + q = 1$$

Alternatively,

$$p = 1 - q \quad OR \quad q = 1 - p$$

All possible combinations of two alleles can be expressed as:

$$(p + q)^2 = 1$$

or more simply,

$$p^2 + 2pq + q^2 = 1$$

In this equation, if p is assumed to be dominant, then p^2 is the frequency of homozygous dominant (AA) individuals in a population, 2pq is the frequency of heterozygous (Aa) individuals, and q^2 is the frequency of homozygous recessive (aa) individuals.

From observations of phenotypes, it is usually only possible to know the frequency of homozygous dominant or recessive individuals, because both dominant and recessives will express the distinguishable traits. However, the Hardy-Weinberg equation allows us to determine the expected frequencies of all the genotypes, if only p or q is known. Knowing p and q, it is a simple matter to plug these values into the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$). This then provides the frequencies of all three genotypes for the selected trait within the population.

This illustration shows Hardy-Weinberg frequency distributions for the genotypes AA, Aa, and aa at all values for frequencies of the alleles, p and q. It should be noted that the proportion of heterozygotes increases as the values of p and q approach 0.5.

Linkage disequilibrum

Linkage is the tendency of genes or DNA sequences (e.g. SNPs) to be inherited together as a consequence of their physical proximity on a single

5   chromosome. The closer together the markers are, the lower the probability that they will be separated during DNA crossing over, and hence the greater the probability that they will be inherited together. Suppose a mutational event introduces a "new" allele in the close proximity of a gene or an allele. The new allele will tend to be inherited together with the alleles present on the "ancestral,"

10   chromosome or haplotype. However, the resulting association, called linkage disequilibrium, will decline over time due to recombination. Linkage disequilibrium has been used to map disease genes. In general, both allele and haplotype frequencies differ among populations. Linkage disequilibrium is varied among the populations, being absent in some and highly significant in others.5

15

Quantification of the relative risk of observable outcomes of a Pharmacogenetics Trial

Let PlaR be the placebo response rate (0% (PlaR (100%) and TntR be the treatment response rate (0% (TntR (100%) of a classical clinical trial. ObsRR is

20   defined as the relative risk between TntR and PlaR:

$$ObsRR = TntR / PlaR.$$

Suppose that in the treatment group there is a polymorphism in relation to drug metabolism such as the treatment response rate is different for each genotypic subgroup of patients. Let q be the allele a frequency of a recessive biallelic locus

25   (e.g. SNP) and $p = 1 - q$ the allele A frequency. Following Hardy-Weinberg equilibrium, the relative frequency of homozygous and heterozygous patients are as follow:

AA: p2   Aa: 2pq   aa: q2

with

30    $(p2+2pq+q2) = 1.$

Let's define AAR, AaR, aaR as respectively the response rates of the AA, Aa and aa patients. We have the following relationship:

$$TntR = AAR*p2 + AaR*2pq + aaR*q2.$$

Suppose that the aa genotypic group of patients has the lowest response rate, i.e. a response rate equal to the placebo response rate (which means that the polymorphism has no impact on natural disease evolution but only on drug action)

5 and let's define ExpRR as the relative risk between AAR and aaR, as

$$ExpRR = AAR / aaR.$$

From the previous equations, we have the following relationships:

$$ObsRR (ExpRR (1/PlaR$$

$$TntR / PlaR = (AAR*p2 + AaR*2pq + aaR*q2) / PlaR$$

10 The maximum of the expected relative risk, max(ExpRR), corresponding to the case of heterozygous patients having the same response rate as the placebo rate, is such that:

$$ObsRR = ExpRR*p2 + 2pq + q2 \quad \Leftrightarrow \quad ExpRR = (ObsRR - 2pq - q2) / p2$$

min(ExpRR),

15 corresponding to the case of heterozygous patients having the same response rate as the homozygous non-affected patients, is such that:

$$ObsRR = ExpRR*(p2 + 2pq) + q2 \quad \Leftrightarrow \quad ExpRR = (ObsRR - q2) / (p2 + 2pq)$$

For example, if q = 0.4, PlaR = 40% and ObsRR = 1.5 (i.e. TntR = 60%), then 1.6 (

20 ExpRR (2.4. This means that the best treatment response rate we can expect in a genotypic subgroup of patients in these conditions would be 95.6% instead of 60%.

This can also be expressed in terms of maximum potential gain between the observed difference in response rates (TntR – PlaR) without any pharmacogenetic hypothesis and the maximum expected difference in response rates

25 (max(ExpRR)*PlaR – TntR) with a strong pharmacogenetic hypothesis:

$$(max(ExpRR)*PlaR - TntR) = [(ObsRR - 2pq - q2) / p2] * PlaR - TntR$$

$$\Leftrightarrow \quad (max(ExpRR)*PlaR - TntR) = [TntR - PlaR*(2pq + q2) - TntR*p2]/p2$$

$$\Leftrightarrow \quad (max(ExpRR)*PlaR - TntR) = [TntR*(1 - p2) - PlaR*(2pq + q2)]/p2$$

$$\Leftrightarrow \quad (max(ExpRR)*PlaR - TntR) = [(1 - p2) / p2] * (TntR - PlaR)$$

30 that is for the previous example, (95.6% – 60%) = [(1 – 0.62)/0.62]* (60% -40%) = 35.6%

Suppose that, instead of one SNP, we have p loci of SNPs for one gene. This means that we have 2p possible haplotypes for this gene and $(2p)(2p-1)/2$ possible genotypes. And with 2 genes with p1 and p2 SNP loci, we have $[(2p1)(2p1-1)/2]*[(2p2)(2p2-1)/2]$ possibilities; and so on. Examining haplotypes instead of

5   combinations of SNPs is especially useful when there is linkage disequilibrium enough to reduce the number of combinations to test, but not complete since in this latest case one SNP would be sufficient. Yet the problem of frequency above still remains with haplotypes instead of SNPs since the frequency of a haplotype cannot be higher than the highest SNP frequency involved.

10

Statistical Methods to be used in Objective Analyses

The statistical significance of the differences between variance frequencies can be assessed by a Pearson chi-squared test of homogeneity of proportions with $n-1$ degrees of freedom. Then, in order to

15   determine whih variance(s) is(are) responsible for an eventual significance, we can consider each variance individually against the rest, up to n comparisons, each based on a 2x2 table. This should result in chi-sequared tests that are individually valid, but taking the most significant of these tests is a form of multiple testing. A Bonferroni's adjustment for multiple testing

20   will thus be made to the P-values, such as $p*=1-(1-p)^n$.

The statistical significance of the difference between genotype frequencies associated to every variance can be assessed by a Pearson chi-squared test of homogeneity of proportions with 2 degrees of freedom, using the same Bonferroni's adjustment as above.

25   Testing for unequal haplotype frequencies between cases and controls can be considered in the same framework as testing for unequal variance frequencies since a single variance can be considered as a haplotype of a single locus. The relevant likelihood ratio test compares a model where two seqarate sets of haplotype frequencies apply to the cases and controls, to

30   one where the entire sample is characterized by a single common set of haplotype frequencies. This can be performed by repeated use of a computer program (Terwilliger and Ott, 1994, Handbook of Human Linkage Analysis,

Baltimore, John Hopkins University Press) to successively obtain the log-likelihood corresponding to the set of haplotpe frequency estimates on the cases ($\ln L_{case}$), on the controls ($\ln L_{control}$), and on the overall ($\ln L_{combined}$). The test statistic $2((\ln L_{case}) + (\ln L_{control}) - (\ln L_{combined}))$ is then chi-squared with $r-1$ degrees of freedom (where r is the number of haplotypes).

To test for potentially confounding effects or effect-modifiers, such as sex, age, etc., logistic regression can be used with case-control status as the outcome variable, and genotypes and covariates (plus possible interactions) as predictor variables.

Example 12   Exemplary Pharmacogenetic Analysis Steps

In accordance with the discussion of distribution frequencies for variances, alleles, and haplotypes, variance detection, and correlation of variances or haplotypes with treatment response variability, the points below list major items which will typically be performed in an analysis of the pharmacogenetic determination of the effects of variances in the treatment of a disease and the selection/optimization of treatment.

- List candidate gene/genes for a known genetic disease, and assign them to the respective metabolic pathways.

- Determine their alleles, observed and expected frequencies, and their relative distributions among various ethnic groups, gender, both in the control and in the study (case) groups

- Measure the relevant clinical/phenotypic (biochemical / physiological) variables of the disease

- If the causal variance/allele in the candidate gene is unknown, then determine linkage disequilibria among variances of the candidate gene(s)

- Divide the regions of the candidate genes into regions of high linkage disequilibrium and low disequilibrium

- Develop haplotypes among variances that show strong linkage disequilibrium using the computation methods.

- Determine the presence of rare haplotypes experimentally. Confirm if the computationally determined rare haplotypes agree with the experimentally determined haplotypes. If there is a disagreement between the experimentally determined haplotypes and the computationally derived haplotypes, drop the computationally derived rare haplotypes,

- Construct cladograms from these haplotypes using the Templeton (1987) algorithm.

- Note regions of high recombination. Divide regions of high recombination further to see patterns of linkage disequilibria.

- Establish association between cladograms and clinical variables using the nested analysis of variance as presented by Templeton (1995), and assign causal variance to a specific haplotype

- For variances in the regions of high recombination, use permutation tests for establishing associations between variances and the phenotypic variables

- If two or more genes are found to affect a clinical variable determine the relative contribution of each of the genes or variances in relation to the clinical variable, using step-wise regression or discriminant function or principal component analysis.

- Determine the relative magnitudes of the effects of any of the two variances on the clinical variable due to their genetic (additive, dominant or epistasis) interaction.

- Using the frequency of an allele or haplotypes, as well as biochemical/clinical variables determined in the *in vitro* or *in vivo* studies, determine the effect of that gene or allele on the expression of the clinical variable, according to the measured genotype approach of Boerwinkle et al (Ann. Hum. Genet 1986).

- Stratify ethnic/ clinical populations based on the presence or absence of a given allele or a haplotype

- Optimize drug dosages based on the frequency of alleles and haplotypes as well as their effects using the measured genotype approach as a guide

Example 13   Method for Producing cDNA

In order to identify sequence variances in a gene by laboratory methods it is in some instances useful to produce cDNA(s) from multiple human subjects. (In other instances it may be preferable to study genomic DNA.). Methods for producing cDNA are known to those skilled in the art, as are methods for amplifying and sequencing the cDNA or portions thereof. An example of a useful cDNA production protocol is provided below. As recognized by those skilled in the art, other specific protocols can also be used.

cDNA Production

** Make sure that all tubes and pipette tips are RNase-free. (Bake them overnight at 100°C in a vaccum oven to make them RNase-free.)

1 Add the following to a RNase-free 0.2 ml micro-amp tube and mix gently:

24 ul water (DEPC treated)
12 ul RNA (1ug/ul)
12 ul random hexamers(50 ng/ul)

2 Heat the mixture to 70°C for ten minutes.

3 Incubate on ice for 1 minute.

4 Add the following:

16 ul 5 X Synthesis Buffer
8 ul 0.1 M DTT
4 ul 10 mM dNTP mix (10 mM each dNTP)
4 ul SuperScript RT II enzyme

Pipette gently to mix.

5 Incubate at 42°C for 50 minutes.

6 Heat to 70°C for ten minutes to kill the enzyme, then place it on ice.

7 Add 160 ul of water to the reaction so that the final volume is 240 ul.

8 Use PCR to check the quality of the cDNA. Use primer pairs that will give a ~800 base pair long piece. See "PCR Optimization" for the PCR protocol.

The following chart shows the reagent amounts for a 20 ul reaction, a 80 ul reaction, and a batch of 39 (which makes enough mix for 36) reactions:

	20 ul X 1 tube	80 ul X 1 tube	80ul X 39 tubes	

water	6 ul	24 ul	936	water
RNA	3 ul	12 ul		RNA
random hexamers	3 ul	12 ul	468	random hexamers
synthesis buffer	4 ul	16 ul	624	synthesis buffer
0.1 M DTT	2 ul	8 ul	312	0.1 M DTT
10mM dNTP	1 ul	4 ul	156	10mM dNTP
SSRT	1 ul	4 ul	156	SSRT

Example 14

Method for Detecting Variances by Single Strand Conformation

5   **Polymorphism (SSCP) Analysis**

This example describes the SSCP technique for identification of sequence variances of genes. SSCP is usually paired with a DNA sequencing method, since the SSCP method does not provide the nucleotide identity of variances. One useful sequencing method, for example, is DNA cycle sequencing of ^{32}P labeled PCR

10 products using the Femtomole DNA cycle sequencing kit from Promega (WI) and the instructions provided with the kit. Fragments are selected for DNA sequencing based on their behavior in the SSCP assay.

Single strand conformation polymorphism screening is a widely used technique for identifying an discriminating DNA fragments which differ from each

15 other by as little as a single nucleotide. As originally developed by Orita et al. (Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci U S A.* 86(8):2766-70, 1989), the technique was used on genomic DNA, however the same group showed that the technique works very well on PCR amplified DNA as well. In the last 10 years the

20 technique has been used in hundreds of published papers, and modifications of the technique have been described in dozens of papers. The enduring popularity of the technique is due to (1) a high degree of sensitivity to single base differences (>90%) (2) a high degree of selectivity, measured as a low frequency of false positives, and (3) technical ease. SSCP is almost always used together with DNA sequencing

25 because SSCP does not directly provide the sequence basis of differential fragment mobility. The basic steps of the SSCP procdure are described below.

When the intent of SSCP screening is to identify a large number of gene variances it is useful to screen a relatively large number of individuals of different racial, ethnic and/or geographic origins. For example, 32 or 48 or 96 individuals is a convenient number to screen because gel electrophoresis apparatus are available with

5   96 wells (Applied Biosystems Division of Perkin Elmer Corporation), allowing 3 X 32, 2 X 48 or 96 samples to be loaded per gel.

The 32 (or more) individuals screened should be representative of most of the worlds major populations. For example, an equal distribution of Africans, Europeans and Asians constitutes a reasonable screening set. One useful source of

10   cell lines from different populations is the Coriell Cell Repository (Camden, NJ), which sells EBV immortalized lyphoblastoid cells obtained from several thousand subjects, and includes the racial/ethnic/geographic background of cell line donors in its catalog. Alternatively, a panel of cDNAs can be isolated from any specific target population.

15   SSCP can be used to analyze cDNAs or genomic DNAs. For many genes cDNA analysis is preferable because for many genes the full genomic sequence of the target gene is not available, however, this circumstance will change over the next few years. To produce cDNA requires RNA. Therefore each cell lines is grown to mass culture and RNA is isolated using an acid/phenol protocol, sold in kit form as

20   Trizol by Life Technologies (Gaithersberg, MD). The unfractionated RNA is used to produce cDNA by the action of a modified Maloney Murine Leukemia Virus Reverse Transcriptase, purchased in kit form from Life Technologies (Superscript II kit). The reverse transcriptase is primed with random hexamer primers to initiate cDNA synthesis along the whole length of the RNAs. This proved useful later in

25   obtaining good PCR products from the 5' ends of some genes. Alternatively, oligodT can be used to prime cDNA synthesis.

Material for SSCP analysis can be prepared by PCR amplification of the cDNA in the presence of one α ^{32}P labeled dNTP (usually α ^{32}P dCTP). Usually the concentration of nonradioactive dCTP is dropped from 200 uM (the standard

30   concentration for each of the four dNTPs) to about 100 uM, and ^{32}P dCTP is added to a concentration of about 0.1-0.3 uM. This involves adding a 0.3- 1 ul (3-10 uCi) of

^{32}P cCTP to a 10 ul PCR reaction. Radioactive nucleotides can be purchased from DuPont/New England Nuclear.

The customary practice is to amplify about 200 base pair PCR products for SSCP, however, an alternative approach is to amplify about 0.8-1.4 kb fragments and

5   then use several cocktails of restriction endonucleases to digest those into smaller fragments of about 0.1-0.4kb, aiming to have as many fragments as possible between .15 and .3 kb. The digestion strategy has the advantage that less PCR is required, reducing both time and costs. Also, several different restriction enzyme digests can be performed on each set of samples (for example 96 cDNAs), and then each of the

10 digests can be run separately on SSCP gels. This redundant method (where each nucleotide is surveyed in three different fragments) reduces both the false negative and false positive rates. For example: a site of variance might lie within 2 bases of the end of a fragment in one digest, and as a result not affect the conformation of that strand; the same variance, in a second or third digest, would likely lie in a location

15 more prone to affect strand folding, and therefore be detected by SSCP.

After digestion, the radiolabelled PCR products are diluted 1:5 by adding formamide load buffer (80% formamide, 1X SSCP gel buffer) and then denatured by heating to 90%C for 10 minutes, and then allowed to renature by quickly chilling on ice. This procedure (both the dilution and the quick chilling) promotes intra- (rather

20 than inter-) strand association and secondary structure formation. The secondary structure of the single strands influences their mobility on nondenaturing gels, presumably by influencing the number of collisions between the molecule and the gel matrix (i.e., gel sieving). Even single base differences consistently produce changes in intrastrand folding sufficient to register as mobility differences on SSCP.

25   The single strands were then resolved on two gels, one a 5.5% acrylamide, 0.5X TBE gel, the other an 8% acrylamide, 10% glycerol, 1X TTE gel. (Other gel recipes are known to those skilled in the art.) The use of two gels provides a greater opportunity to recognize mobility differences. Both glycerol and acrylamide concentration have been shown to influence SSCP performance. By routinely

30 analyzing three different digests under two gel conditions (effectively 6 conditions), and by looking at both strands under all 6 conditions, one can achieve a 12-fold

sampling of each base pair of cDNA. However, if the goal is to rapidly survey many genes or cDNAs then a less redundant procedure would be optimal.

Example 15

5 **Method for Detecting Variances by T4 endonuclease VII (T4E7) mismatch cleavage method**

The enzyme T4 endonuclease VII is derived from the bacteriophage T4. T4 endonuclease VII is used by the bacteriophage to cleave branched DNA intermediates which form during replication so the DNA can be processed and

10 packaged. T4 endonuclease can also recognize and cleave heteroduplex DNA containing single base mismatches as well as deletions and insertions. This activity of the T4 endonuclease VII enzyme can be exploited to detect sequence variances present in the general population.

15 The following are the major steps involved in identifying sequence variations in a candidate gene by T4 endonuclease VII mismatch cleavage:

1. Amplification by the polymerase chain reaction (PCR) of 400-600 bp regions of the candidate gene from a panel of DNA samples The DNA samples can

20 either be cDNA or genomic DNA and will represent some cross section of the world population.

2. Mixing of a fluorescently labeled probe DNA with the sample DNA. Heating and cooling the mixtures causing heteroduplex formation between the probe DNA and the sample DNA.

25 3. Addition of T4 endonuclease VII to the heteroduplex DNA samples. T4 endonuclease will recognize and cleave at sequence variance mismatches formed in the heteroduplex DNA.

4. Electrophoresis of the cleaved fragments on an ABI sequencer to determine the site of cleavage.

30 5. Sequencing of a subset of PCR fragments identified by T4 endonuclease VI to contain variances to establish the specific base variation at that location.

A more detailed description of the procedure is as follows:

A candidate gene sequence is downloaded from an appropriate database. Primers for PCR amplification are designed which will result in the target sequence being divided into amplification products of between 400 and 600 bp. There will be a minimum of a 50 bp of overlap not including the primer sequences between the 5' and 3' ends of adjacent fragments to ensure the detection of variances which are located close to one of the primers.

Optimal PCR conditions for each of the primer pairs is determined experimentally. Parameters including but not limited to annealing temperature, pH, $MgCl_2$ concentration, and KCl concentration will be varied until conditions for optimal PCR amplification are established. The PCR conditions derived for each primer pair is then used to amplify a panel of DNA samples (cDNA or genomic DNA) which is chosen to best represent the various ethnic backgrounds of the world population or some designated subset of that population.

One of the DNA samples is chosen to be used as a probe. The same PCR conditions used to amplify the panel are used to amplify the probe DNA. However, a flourescently labeled nucleotide is included in the deoxy-nucleotide mix so that a percentage of the incorporated nucleotides will be fluorescently labeled.

The labeled probe is mixed with the corresponding PCR products from each of the DNA samples and then heated and cooled rapidly. This allows the formation of heteroduplexes between the probe and the PCR fragments from each of the DNA samples. T4 endonuclease VII is added directly to these reactions and allowed to incubate for 30 min. at 37 C. 10 ul of the Formamide loading buffer is added directly to each of the samples and then denatured by heating and cooling. A portion of each of these samples is electrophoresed on an ABI 377 sequencer. If there is a sequence variance between the probe DNA and the sample DNA a mismatch will be present in the heteroduplex fragment formed. The enzyme T4 endonuclease VII will recognize the mismatch and cleave at the site of the mismatch. This will result in the appearance of two peaks corresponding to the two cleavage products when run on the ABI 377 sequencer.

Fragments identified as containing sequencing variances are subsequently sequenced using conventional methods to establish the exact

location and sequence variance.

Example 16

5   **Method for Detecting Variances by DNA sequencing.**

Sequencing by the Sanger dideoxy method or the Maxim Gilbert chemical cleavage method is widely used to determine the nucleotide sequence of genes. Presently, a worldwide effort is being put forward to sequence the entire human genome. The Human Genome Project as it is called has already resulted in the

10 identification and sequencing of many new human genes. Sequencing can not only be used to identify new genes, but can also be used to identify variations between individuals in the sequence of those genes.

The following are the major steps involved in identifying sequence variations in a candidate gene by sequencing:

15

1.   Amplification by the polymerase chain reaction (PCR) of 400-700 bp regions of the candidate gene from a panel of DNA samples The DNA samples can either be cDNA or genomic DNA and will represent some cross section of the world population.

20 2.   Sequencing of the resulting PCR fragments using the Sanger dideoxy method. Sequencing reactions are performed using flourescently labeled dideoxy terminators and electrophoresedon an ABI 377 sequencer or its equivalent.

3.   Analysis of the resulting data from the ABI 377 sequencer using software

25   programs designed to identify sequence variations between the different samples analyzed.

A more detailed description of the procedure is as follows:

A candidate gene sequence is downloaded from an appropriate database.

30 Primers for PCR amplification are designed which will result in the target sequence being divided into amplification products of between 400 and 700 bp. There will be a minimum of a 50 bp of overlap not including the primer sequences between the 5'

and 3' ends of adjacent fragments to ensure the detection of variances which are located close to one of the primers.

Optimal PCR conditions for each of the primer pairs is determined experimentally. Parameters including but not limited to annealing temperature, pH,
5 MgCl$_2$ concentration, and KCl concentration will be varied until conditions for optimal PCR amplification are established. The PCR conditions derived for each primer pair is then used to amplify a panel of DNA samples (cDNA or genomic DNA) which is chosen to best represent the various ethnic backgrounds of the world population or some designated subset of that population.

10 PCR reactions are purified using the QIAquick 8 PCR purification kit (Qiagen cat# 28142) to remove nucleotides, proteins and buffers. The PCR reactions are mixed with 5 volumes of Buffer PB and applied to the wells of the QIAquick strips. The liquid is pulled through the strips by applying a vacuum. The wells are then washed two times with 1 ml of buffer PE and allowed to dry for 5
15 minutes under vacuum. The PCR products are eluted from the strips using 60 ul of elution buffer.

The purified PCR fragments are sequenced in both directions using the Perkin Elmer ABI Prism™ Big Dye™ terminator Cycle Sequencing Ready Reaction Kit (Cat# 4303150). The following sequencing reaction is set up: 8.0 ul Terminator
20 Ready Reaction Mix, 6.0 ul of purified PCR fragment, 20 picomoles of primer, deionized water to 20 ul. The reactions are run through the following cycles 25 times: 96°C for 10 second, annealing temperature for that particular PCR product for 5 seconds, 60°C for 4 minutes.

The above sequencing reactions are ethanol precipitated directly in the PCR
25 plate, washed with 70% ethanol, and brought up in a volume of 6 ul of formamide dye. The reactions are heated to 90°C for 2 minutes and then quickly cooled to 4°C. 1 ul of each sequencing reaction is then loaded and run on an ABI 377 sequencer.

The output for the ABI sequencer appears as a series of peaks where each of the different nucleotides, A, C, G, and T appear as a different color. The nucleotide
30 at each position in the sequence is determined by the most prominent peak at each location. Comparison of each of the sequencing outputs for each sample can be examined using software programs to determine the presence of a variance in the

sequence.   One example of heterozygote detection using sequencing with dye labeled terminators is described by Kwok *et. al.* (Kwok, P.-Y.; Carlson, C.; Yager, T.D., Ankener, W.,and D. A. Nickerson, *Genomics* 23, 138-144, 1994).   The software compares each of the normalized peaks between all the samples base by

5   base and looks for a 40% decrease in peak height and the concomitant appearance of a new peak underneath.   Possible variances flagged by the software are further analyzed visually to confirm their validity

In connection with the provision and description of nucleic acid sequences,

10   the references herein to gene names and to GenBank and OMIM reference numbers provides the relevant sequences, recognizing that the described sequences will, in most cases, also have other corresponding allelic variants.   Also, it is recognized that the referenced sequences may contain sequencing error.   Such error does not interfere with identification of a relevant gene or portion of a gene, and can be

15   readily corrected by redundant sequencing of the relevant sequence (preferably using both strands of DNA).   Nucleic acid molecules or sequences can be readily obtained or determined utilizing the reference sequences.   In general, molecules such as nucleic acid hybridization probes and amplification primers can be provided and are described by the selected portion of the reference sequence, corrected if necessary.

20   Thus, nucleic acid hybridization probes and/or primers are thus described by a portion of a reference sequence or a sequence complementary thereto (sequence corrected if necessary), or an allelic variant of such a sequence, which preferably includes at least one variance site, preferably a variance site indicative of the effectiveness of a treatment for a disease or condition, and preferably include at least

25   12,13,14,15,16,17,18,19,20,23,25,27,30,35,40,45, or 50 nucleotides.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains.   All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

30   One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein.   The methods, variances, and compositions

described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

5   It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. For example, using other compounds, and/or methods of administration are all within the scope of the present invention. Thus, such additional embodiments are within the scope of the present invention and

10   the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be

15   replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus,

20   it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

25   In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Thus, additional embodiments are within the scope of the invention and

30   within the following claims.

Table 10

Variance Table

Name	GID	OMIM_ID	VGX_Symbol	Description
	Variance_Start	Variance	CDS_Context	

D13811 D13811 238310　　GEN-AA　Glycine cleavage system: Protein T
　277　148G>T　V50L

D13811 D13811 238310　　GEN-AA　Glycine cleavage system: Protein T
　1073　944G>A　R315K

D13811 D13811 238310　　GEN-AA　Glycine cleavage system: Protein T
　1083　954G>A　S

D13811 D13811 238310　　GEN-AA　Glycine cleavage system: Protein T
　1773　1644C>T　3

D13811 D13811 238310　　GEN-AA　Glycine cleavage system: Protein T
　2037　1908C>T　3

J03626 J03626 258900　　GEN-C6　Uridine monophosphate synthetase
(orotate phosphoribosyl transferase and orotidine-5-decarboxylase)　742　638G>C
　G213A

J03626 J03626 258900　　GEN-C6　Uridine monophosphate synthetase
(orotate phosphoribosyl transferase and orotidine-5-decarboxylase)　1575　1471A>G
　3

J03626 J03626 258900　　GEN-C6　Uridine monophosphate synthetase
(orotate phosphoribosyl transferase and orotidine-5-decarboxylase)　1424　1320C>T
　S

J04031 J04031 None　GEN-CB　　Methenyltetrahydrofolate cyclohydrolase
　454　401G>A　R134K

J04031 J04031 None　GEN-CB　　Methenyltetrahydrofolate cyclohydrolase
　969　916C>G　Q306E

J04031 J04031 None　GEN-CB　　Methenyltetrahydrofolate cyclohydrolase
　1614　1561T>C　S

J04031 J04031 None　GEN-CB　　Methenyltetrahydrofolate cyclohydrolase
　2011　1958G>A　R653Q

J04031 J04031 None　GEN-CB　　Methenyltetrahydrofolate cyclohydrolase
　2335　2282C>T　T761M

Name			VGX_Symbol	Description	Variance_Start	Variance
K02581 K02581 188300			GEN-CI	Thymidine kinase 1	90	33C>T S
K02581 K02581 188300 S			GEN-CI	Thymidine kinase 1	279	222G>A
K02581 K02581 188300 S			GEN-CI	Thymidine kinase 1	282	225G>A
K02581 K02581 188300 3			GEN-CI	Thymidine kinase 1	772	715A>G
K02581 K02581 188300 3			GEN-CI	Thymidine kinase 1	867	810G>A
K02581 K02581 188300 P141L			GEN-CI	Thymidine kinase 1	479	422C>T
K02581 K02581 188300 G19R			GEN-CI	Thymidine kinase 1	112	55G>A
K02581 K02581 188300 E144K			GEN-CI	Thymidine kinase 1	487	430G>A
K02581 K02581 188300 R130G			GEN-CI	Thymidine kinase 1	445	388A>G

K02581K02581188300   GEN-CI   Thymidine kinase 1   313   256C>T
    F

K02581K02581188300   GEN-CI   Thymidine kinase 1   334   277G>T
    V93F

K02581K02581188300   GEN-CI   Thymidine kinase 1   329   272-
278TGGCTGT>TGGCTGT   S

M64590   M64590   238300   GEN-FU   Glycine cleavage system:
Protein P   3076   2926A>G   M976V

M69175   M69175   None   GEN-FX   Glycine cleavage system: Protein
H   710   686C>G   3

M69175   M69175   None   GEN-FX   Glycine cleavage system: Protein
H   1007   983C>T   3

U09178U09178274270   GEN-HA   Dihydropyrimidine Dehydrogenase
    166   85T>C   C29R

U09178U09178274270   GEN-HA   Dihydropyrimidine Dehydrogenase
    577   496A>G   M166V

U09178U09178274270   GEN-HA   Dihydropyrimidine Dehydrogenase
    3925   3844A>G   3

U09178U09178274270   GEN-HA   Dihydropyrimidine Dehydrogenase
    3937   3856T>C   3

U09178U09178274270   GEN-HA   Dihydropyrimidine Dehydrogenase
    1708   1627A>G   I543V

U09178U09178274270   GEN-HA   Dihydropyrimidine Dehydrogenase
    3432   3351T>C   3

U09178U09178274270   GEN-HA   Dihydropyrimidine Dehydrogenase
    3730   3649G>A   3

U09178U09178274270   GEN-HA   Dihydropyrimidine Dehydrogenase
    638   557A>G   Y186C

U19720U19720600424   GEN-I1Folate Transporter (SLC19A1)   341   246C>G
S

U19720U19720600424   GEN-I1Folate Transporter (SLC19A1)   53   (-43)T>C
5

U19720U19720600424   GEN-I1Folate Transporter (SLC19A1)   175   80G>A
R27H

U19720U19720600424   GEN-I1Folate Transporter (SLC19A1)   791   696C>T
S

U50929U50929None   GEN-JF   Methionine synthetase (aka homocysteine
methyltransferase)   2017   1991A>G   3

U77088U77088None   GEN-K4   Thymidine kinase 2   1480   1472T>C   3

X02308X02308188350   GEN-KL   Thymidylate synthetase 1066   961T>C
3

X02308X02308188350   GEN-KL   Thymidylate synthetase 1136   1031A>G
3

X02308X02308188350   GEN-KL   Thymidylate synthetase 1497   1392T>A
3

X59543X59543None   GEN-M2   Ribonucleoside diphosphate reductase   1037
    850C>A   S

X59543X59543None   GEN-M2   Ribonucleoside diphosphate reductase   2410
    2223G>A   S

X59543X59543None   GEN-M2   Ribonucleoside diphosphate reductase   2419
    2232A>G   S

X59543X59543None   GEN-M2   Ribonucleoside diphosphate reductase   2717
    2530T>A   3

X59618 X59618 180390   GEN-M3   Ribonucleotide reductase M2 polypeptide
           524   330C>G   S
X59618 X59618 180390   GEN-M3   Ribonucleotide reductase M2 polypeptide
          1636   1442C>T   3
X59618 X59618 180390   GEN-M3   Ribonucleotide reductase M2 polypeptide
          2259   2065T>C   3
X59618 X59618 180390   GEN-M3   Ribonucleotide reductase M2 polypeptide
           189   (-6)T>G   5
X90858 X90858 None   GEN-NQ   Uridine phosphorylase 1133   781T>A
C261S
X17620 X17620 None   GEN-20M   Human mRNA for Nm23 protein, involved in
developmental regulation (homolog. to Drosophila Awd protein) 244   244G>T
D82Y
L38928 L38928 None   GEN-2PT   Homo sapiens 5,10-methenyltetrahydrofolate
synthetase mRNA, complete cds   617   604A>G   T202A
S72487 S72487 None   GEN-3LD   orf1 5 to PD-ECGF/TP...orf2 5 to PD-ECGF/TP
[human, epidermoid carcinoma cell line A431, mRNA, 3 genes, 1718 nt] 601   437G>C
3
M98045   M98045   None   GEN-4C3   Homo sapiens folylpolyglutamate
synthetase mRNA, complete cds   1747   1677G>T   3
M98045   M98045   None   GEN-4C3   Homo sapiens folylpolyglutamate
synthetase mRNA, complete cds   1900   1830T>C   3
L11931 L11931 None   GEN-4DT   Human cytosolic serine hydroxymethyltransferase
(SHMT) mRNA, complete cds 1444   1420C>T   L474F
L11931 L11931 None   GEN-4DT   Human cytosolic serine hydroxymethyltransferase
(SHMT) mRNA, complete cds 1541   1517C>T   3
DHFR J00140 126060   GEN-4E9   Human dihydrofolate reductase gene
           721   679T>A   3
DHFR J00140 126060   GEN-4E9   Human dihydrofolate reductase gene
           829   787C>T   3
U09806 U09806 None   GEN-4FZ   Human methylenetetrahydrofolate reductase
mRNA, partial cds   1289   1289C>A   E430A
U09806 U09806 None   GEN-4FZ   Human methylenetetrahydrofolate reductase
mRNA, partial cds   473   473G>A   R158Q
U09806 U09806 None   GEN-4FZ   Human methylenetetrahydrofolate reductase
mRNA, partial cds   550   550C>T   F
U09806 U09806 None   GEN-4FZ   Human methylenetetrahydrofolate reductase
mRNA, partial cds   668   668C>T   A223V
U09806 U09806 None   GEN-4FZ   Human methylenetetrahydrofolate reductase
mRNA, partial cds   1308   1308T>C   3
U09806 U09806 None   GEN-4FZ   Human methylenetetrahydrofolate reductase
mRNA, partial cds   120   120T>C   S
U09806 U09806 None   GEN-4FZ   Human methylenetetrahydrofolate reductase
mRNA, partial cds   1059   1059T>C   S

Claims

What we claim is:

5   1.   A method for selecting a treatment for a patient suffering from a condition or
disease, comprising

determining whether cells of said patient contain at least one variance of a
gene, wherein the presence or the absence of said variance in said cells is indicative
of the effectiveness of said treatment for said condition or disease,

10   wherein said gene is a folate transport or metabolism gene or a pyrimidine
transport or metabolism gene.

2.   The method of claim 1, wherein said gene is selected from the group
consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate

15   Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase.
Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase,
Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase,
Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-
drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate

20   dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase,
Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine
transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T,
Protein L,_Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside
transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine

25   phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase,
Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase
M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A
subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase,
Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase,

30   β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate
synthase.

3.   The method of claim 1, wherein the presence of said at least one variance is
indicative that said treatment will be effective for said patient.

35

4.   The method of claim 1, wherein the presence of said variance is indicative
that said treatment will be ineffective or contra-indicated for said patient.

5. The method of claim 1, wherein said at least one variance comprises a plurality of variances.

6. The method of claim 5, wherein said plurality of variances comprise a haplotype or haplotypes.

7. The method of claim 1, wherein said selecting a treatment further comprises identifying a compound differentially active on a form of said gene containing said at least one variance.

8. The method of claim 1, wherein said compound is selected from the group consisting of a reduced folate, a folate analog, folic acid, a fluoropyrimidine, a dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor.

9. The method of claim 1, wherein said selecting a treatment further comprises eliminating a treatment, wherein said presence or absence of said at least one variance is indicative that said treatment will be ineffective or contra-indicated.

10. The method of claim 1, wherein said treatment comprises a first treatment and a second treatment, said method comprising the steps of:
    identifying a said first treatment effective to treat said disease or condition; and
    identifying a said second treatment which reduces a deleterious effect of said first treatment.

11. The method of claim 1, wherein said selecting a treatment further comprises selecting the method of administration of a compound effective to treat said disease, wherein said presence or absence of said at least one variance is indicative of the appropriate method of administration for said compound.

12. The method of claim 11, wherein said selecting the method of administration comprises selecting a suitable dosage level or frequency of administration of a compound.

13. The method of claim 1, further comprising determining the level of expression of said gene or the level of activity of a protein containing a polypeptide expressed from said gene,

wherein the combination of the determination of the presence or absence of

5   said at least one variance and the determination of the level of activty or the level of expression provides a further indication of the effectiveness of said treatment.

14. The method of claim 1, wherein said disease or condition is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate

10   deficiency, cardiovascular disease, transplantation, and spina bifida.

15. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises amplifying a segment of nucleic acid including at least one of said variances.

15

16. The method of claim 15, wherein said segment of nucleic acid is 500 nucleotides or less in length.

17. The method of claim 15, wherein said segment of nucleic acid is 100

20   nucleotides or less in length.

18. The method of claim 15, wherein said segment of nucleic acid is 45 nucleotides or less in length.

25   19. The method of claim 15, wherein said segment includes a plurality of variances.

20. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises contacting nucleic acid comprising a variance

30   site with at least one nucleic acid probe, wherein said at least one probe preferentially hybridizes with a nucleic acid sequence including said variance site and containing a complementary base at said variance site under selective hybridization conditions.

35   21. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises sequencing at least one nucleic acid sequence.

22. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises mass spectrometric determination of at least one nucleic acid sequence.

23. The method of claim 1, wherein the detection of the presence or absence of said at least one variance comprises determining the haplotype of a plurality of variances in a gene.

24. A method for selecting a method of treatment, comprising comparing at least one variance in at least one gene in a patient suffering from a disease or condition with a list of variances in said at least one gene indicative of the effectiveness of at least one method of treatment, wherein said at least one gene is a folate transport or metabolism gene or a pyrimidine transport or metabolism gene.

25. The method of claim 24, wherein said gene is selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L,_Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase.

26.   The method of claim 24, wherein said at least one variance comprises a plurality of variances.

27.   The method of claim 24, wherein said list of variances comprises a plurality
5   of variances.

28.   The method of claim 24, wherein at least one said method of treatment comprises the administration of a compound effective against said disease or condition to a patient.
10
29.   The method of claim 28, wherein said compound is selected from the group consisting of reduced folate, a folate analog, folic acid, a fluoropyrimidine, a dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor.
15
30.   The method of claim 24, wherein the presence or absence of at least one variance in said gene is indicative that said treatment will be effective in said patient.

31.   The method of claim 24, wherein the presence or absence of at least one
20   variance in said gene is indicative that said treatment will be ineffective or contra-indicated.

32.   The method of claim 24, wherein said treatment is a first treatment and the presence or absence of at least one variance in said gene is indicative that a second
25   treatment will be beneficial to reduce a deleterious effect of said first treatment.

33.   The method of claim 24, wherein said at least one method of treatment is a plurality of methods of treatment.

30   34.   The method of claim 33, wherein said selecting comprises determining whether any of said plurality of methods of treatment will be more effective than at least one other of said plurality of methods of treatment.

35.   The method of claim 24, wherein said disease is selected from the group
35   consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida.

36.   A method for selecting a method of administration to a patient suffering from a condition or disease for a compound or compounds effective to treat said condition or disease, comprising the step of

5   determining whether at least one variance in a gene is present or absent in cells of said patient, wherein said presence or absence of said at least one variance is indicative of an appropriate method of administration for said compound, and

        wherein said gene is a folate transport or metabolism or pyridine transport or metabolism gene.

10

37.   The method of claim 36, wherein said gene is selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase,

15   Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine

20   transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, ·Protein L‗Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase

25   M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase.

30

38.   The method of claim 36, wherein said selecting a method of administration comprises selecting a dosage level or frequency or frequency of administration of said compound.

35   39.   The method of claim 36, wherein said drug is selected from the group consisting of reduced folate, a folate analog, folic acid, a fluoropyrimidine, a

dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor.

40.   The method of claim 36, wherein said disease is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida .

41.   A method for selecting a patient for administration of a method of treatment, comprising
        comparing the presence or absence of at least one variance in a gene in cells of a patient suffering from a disease or condition with a list of variances in said gene, wherein the presence or absence of said at least one variance in said cells is indicative that said treatment will be effective in said patient; and
        determining whether said patient will receive said method of treatment based on the presence or absence of said at least one variance in said cells,
        wherein said gene is a folate transport or metabolism gene or a pyrimidine transport or metabolism gene.

42.   The method of claim 41, wherein said gene is selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase,

β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

43.   The method of claim 41, wherein said method of treatment comprises administration of a compound effective against said disease or condition.

44.   The method of claim 43, wherein said disease is selected from the group consisting of reduced folate, a folate analog, folic acid, a fluoropyrimidine, a dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor .

45.   The method of claim 41, wherein said determining comprises assigning said patient to a group to receive said method of treatment or to a control group.

46.   A method for identifying the presence or absence of at least one form of a gene in cells of an individual, comprising the steps of:
       a)   determining the presence or absence of at least one variance in said gene in said cells, wherein said gene is a folate transport or metabolism or pyrimidine transport or metabolism gene.

47.   The method of claim 46, wherein said gene is selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase,

Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

5   48.   The method of claim 46, wherein said individual suffers from a disease or condition.

      49.   The method of claim 46, wherein the presence or absence of said at least one variance is indicative of the effectiveness of a therapeutic treatment in a patient
10   having cells containing said at least one variance.

      50.   The method of claim 46, wherein said determining comprises amplifying a segment of nucleic acid including a site of at least one of said at least one variance.

15   51.   The method of claim 46, wherein said determining comprises contacting a nucleic acid sequence containing a variance site corresponding to a said variance with a probe which specifically binds under selective binding conditions to a nucleic acid sequence comprising at least one said variance.

20   52.   The method of claim 46, wherein the detection of the presence or absence of said at least one variance comprises sequencing at least one nucleic acid sequence.

      53.   The method of claim 46, wherein the detection of the presence or absence of said at least one variance comprises mass spectrometric determination of at least one
25   nucleic acid sequence.

      54.   The method of claim 46, wherein the detection of the presence or absence of said at least one variance comprises determining the haplotype of a plurality of variances in a gene.
30

      55.   A pharmaceutical composition comprising
              a compound which has a differential effect in patients having at least one copy of a particular form of a gene, wherein said gene is a folate transport or
35   metabolism gene or a pyrimidine transport or metabolism gene; and
              a pharmaceutically acceptable carrier or excipient or diluent,

wherein said composition is adapted to be preferentially effective to treat a patient with cells comprising a form of said gene comprising at least one variance.

56.   The composition of claim 55, wherein said gene is selected from the group
5   consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-
10   drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L,_Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside
15   transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase,
20   Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

57.   The compositon of claim 55, wherein said patient suffers from a disease or
25   condition selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida .

58.   The pharmaceutical composition of claim 55, wherein said pharmaceutical
30   composition is subject to a regulatory limitation restricting the use of said pharmaceutical composition to patients having at least one copy of a form of a gene comprising at least one variance.

59.   The pharmaceutical composition of claim 55, wherein said pharmaceutical
35   composition is subject to a regulatory limitation indicating said pharmaceutical composition is not to be used in patients having at least one copy of a form of a gene comprising at least one variance.

60.   The pharmaceutical composition of claim 55, wherein said pharmaceutical
composition is packaged, and the packaging includes a label or insert restricting the
use of said pharmaceutical composition to patients having at least one copy of a

5   form of a gene comprising at least one variance.

61.   The pharmaceutical composition of claim 55, wherein said pharmaceutical
composition is packaged, and said packaging includes a label or insert requiring the
use of a test to determine the presence or absence of at least one variance in cells of a

10   said patient.

62.   A probe which specifically binds under selective binding conditions to a
nucleic acid sequence comprising at least one variance in a gene selected from the

15   group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor
(γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate
synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase,
Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase,
Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-

20   drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate
dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase,
Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine
transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T,
Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside

25   transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine
phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase,
Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase
M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A
subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase,

30   Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase,
β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate
synthase .

63.   The probe of claim 62, wherein said probe comprises a nucleic acid sequence
35   500 nucleotide bases or fewer in length.

64.   The probe of claim 62, wherein said nucleic acid sequence is 100 or fewer nucleotide bases in length.

65.   The probe of claim 62, wherein said nucleic acid sequence is 25 or fewer nucleotide bases in length.

66.   The probe of claim 62, wherein said probe comprises DNA.

67.   The probe of claim 62, wherein said probe comprises DNA and at least one nucleic acid analog.

68   The probe of claim 62, wherein said probe comprises peptide nucleic acid (PNA

69.   The probe of claim 62, further comprising a detectable label.

70.   The probe of claim 69, wherein said detectable label is a fluorescent label.

71.   A method for determining a genotype of an individual, comprising analyzing at least one nucleic acid sequence from cells of said individual using mass spectrometric analysis,
        wherein said nucleic acid sequence is a portion of a folate transport or metabolism gene or pyrimidine transport or metabolism gene or a complementary sequence.

72.   The method of claim 71, wherein said analyzing a nucleic acid sequence comprises determining the presence or absence of a variance in said gene.

73.   The method of claim 71, wherein said analyzing a nucleic acid sequence comprises determining the nucleotide sequence of said at least one nucleic acid sequence.

74.   The method of claim 71, wherein said at least one nucleic acid sequence is 500 nucleotides or less in length.

75. The method of claim 71, wherein said at least one nucleic acid sequence comprises at least one variance site in said gene.

5   76. An isolated, purified or enriched nucleic acid sequence of 15 to 500 nucleotides in length, comprising at least one variance, wherein said sequence has the base sequence of a portion of an allele of a gene selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase.
10   Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase,
15   Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L,Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase,
20   Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate
25   synthase or a sequence complementary thereto.

77. The nucleic acid sequence of claim 76, wherein said nucleic acid sequence is 15 to 100 nucleotide bases in length.

30   78. The nucleic acid sequence of claim 76, wherein said nucleic acid sequence sequence is 15 to 25 nucleotide bases in length.

79. A method for determining whether a compound has differential effects on
35   cells containing at least one different form of a folate transport or metabolism or pyridine transport or metabolism gene, comprising the steps of:

contacting a first cell and a second cell with said compound, wherein said first cell and said second cell differ in the presence or absence of at least one variance in said gene; and

determining whether the response of said first cell and said second cell to
5 said compound differ, wherein the difference in said response is due to the presence or absence of said at least one variance.

80. The method of claim 79, wherein said gene is selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate
10 Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate
15 dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine
20 phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase,
25 β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

81. The method of claim 79, wherein at least one of said first cell and said second cell are contacted *in vivo*.
30
82. The method of claim 79, wherein at least one of said first cell and said second cell are contacted *in vitro*.

83. The method of claim 81, wherein at least one of said first cell and said
35 second cell is contacted *in vivo* in a plurality of patients suffering from a disease or condition

84.   A method of treating a patient suffering from a condition or disease, comprising the steps of:

a)   determining whether cells of said patient contain a form of a gene which comprises at least one variance, wherein the presence or absence of said at least one variance is indicative that a treatment will be effective in said patient; and

b)   administering said treatment to said patient.

85.   The method of claim 84, wherein said gene is à folate transport or metabolism gene or a pyrimidine transport or metabolism gene. .

86.   The method of claim 84, wherein said gene is selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L,Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase, Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

87.   The method of claim 84, wherein said disease is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida .

88.   The method of claim 84, wherein the presence of said at least one variance is indicative that said treatment will be effective in said patient.

89.   The method of claim 88, wherein said treatment comprises the administration of a compound preferentially active for said condition or disease in a said patient having said at least one variance in said gene.

90.   The method of claim 89, wherein said compound is selected from the group consisting of reduced folate, a folate analog, folic acid, a fluoropyrimidine, a dihydropyrimidine dehydrogenase inhibitor, a cytidine analog, a pyrimidine analog, a ribonucletide reductase inhibitor, and a nucleotide/nucleoside uptake inhibitor .

91.   The method of claim 84, wherein the presence of said at least one variance in said gene is indicative of an appropriate dosage or frequency of administration of a compound in said treatment.

92.   A method of treating a patient suffering from a disease or condition, comprising the steps of:
        a)   comparing the presence or absence of at least one variance in at least one gene in cells of a patient suffering from a disease or condition with a list of variances in said at least one gene indicative of the effectiveness of at least one method of treatment;
        b)   selecting a method of treatment from said at least one method of treatment, wherein the presence or absence of at least one of said at least one variance is indicative that said method of treatment will be effective in said patient; and
        c)   administering said method of treatment to said patient.

93.   The method of claim 92, wherein said at least one gene comprises a folate transport or metabolism or pyrimidine transport or metabolism gene.

94.   The method of claim 92, wherein said gene is selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate

dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L, Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside

5   transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase,

10   Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

95.   The method of claim 92, further comprising determining the presence or
15   absence of said at least one variance in cells of said patient.

96.   The method of claim 92, wherein said at least one variance comprises a plurality of variances.

20   97.   The method of claim 92, wherein said list of variances comprises a plurality of variances.

98.   The method of claim 97, wherein said plurality of variances comprises a haplotype or haplotypes.

25

99.   The method of claim 92, wherein said method of treatment comprises the administration of a compound effective against said disease or condition.

100.   The method of claim 92, wherein said treatment is a first treatment and the
30   presence or absence of at least one variance in said gene is indicative that a second treatment will be beneficial to reduce a deleterious effect of said first treatment.

101.   The method of claim 92, wherein said at least one method of treatment is a plurality of methods of treatment.

35

102.   The method of claim 92, wherein said disease or condition is selected from the group consisting of cancer, proliferative skin diseases, autoimmune diseases, folate deficiency, cardiovascular disease, transplantation, and spina bifida .

5

103.   A method of treating a patient suffering from a disease or condition, comprising the steps of:

a)   comparing the presence or absence of at least one variance in at least one gene in cells of a patient suffering from a disease or condition with a list of

10   variances in said at least one gene indicative of the effectiveness of at least one method of treatment;

b)   eliminating a method of treatment from said at least one method of treatment, wherein the presence or absence of at least one of said at least one variance is indicative that said method of treatment will be ineffective or contra-

15   indicated in said patient;

c)   selecting an alternative method of treatment effective to treat said disease or condition; and

e.   administering said alternative method of treatment to said patient.

20   104.   The method of claim 103, further comprising determining the presence or absence of said at least one variance in cells of said patient.

105.   The method of claim 103, wherein said at least one gene comprises a folate transport or metabolism or pyrimidine transport or metabolism gene.

25

106.   The method of claim 103, wherein said gene is selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase,

30   Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase, Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine

35   transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T, Protein L,_Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine

phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase, Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase,

5 Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase, β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate synthase .

10 107. A method for producing a pharmaceutical composition, comprising the steps of:

a) identifying a compound which has differential activity against a disease or condition in patients having at least one variance in a gene;

b) compounding said pharmaceutical composition by combining said

15 compound and a pharmaceutically acceptable carrier or excipient or diluent in manner adapted to be preferentially effective in patients having said at least one variance.

20 108. A method for producing a pharmaceutical agent, comprising the steps of:

a) identifying a compound which has differential activity against a disease or condition in patients having at least one variance in a gene;

b) synthesizing said compound in an amount sufficient to provide a pharmaceutical effect in a patient suffering from said disease or condition.

25

109. A method for determining whether a variance in a gene provides variable patient response to a method of treatment for a disease or condition, comprising the steps of:

30 determining whether the response of a first patient or set of patients suffering from a disease or condition differs from the response of a second patient or set of patients suffering from said disease or condition;

determining whether the presence or absence of at least one variance in at least one folate transport or metabolism gene or pyrimidine transport or metabolism

35 gene differs between said first patient or set of patient and said second patient or set of patients;

wherein correlation of said presence or absence of at least one variance and the response of said patient to said treatment is indicative that said at least one variance provides variable patient response.

5   110.   The method of claim 109, further comprising identifying at least one variance in a said gene.

111.   The method of claim 109, wherein a plurality of pairwise comparisons of treatment response and the presence or absence of at least one variance are

10 performed for a plurality of patients.

112.   The method of claim 109, wherein said determining whether the presence or absence of at least one variance in at least one gene comprises comparing the response of at least one patient homozygous for said at least one variance with at

15 least one patient homogyzous for the alternative form of said at least one variance.

113.   The method of claim 109, wherein said determining whether the presence or absence of said at least one variance in at least one gene comprises comparing the response of at least one patient heterogyzous for said at least one variance with the

20 response of at least one patient homozygous for said at least one variance.

114.   The method of claim 109, wherein it is previously known that patient response to said method of treatment is variable.

25 115.   The method of claim 109, wherein said gene is selected from the group consisting of Folate receptor 1(α),Folate receptor (β),Folate receptor (γ),Folate Transporter, Pteroyl-γ-glutamyl carboxypeptidase, Folylpolyglutamate synthetase. Thymidylate synthase, Formiminotetrahy-drofolate cyclodeaminase, Methenyltetrahy-drofolate synthetase, Methylenetetrahy-drofolate dehydrogenase,

30 Methionine synthetase, Dihydrofolate reductase, Methenyltetrahy-drofolate cyclohy-drolase; formylte-trahydrofolate synthetase; Meth-enyltetrahydrofol-ate dehydrogenase, Glutamate form-iminotransferase, Formyltetrahydrofolate hydrolase, Methylenetetrahydrofolate synthase, Methylenetetrahydrofolate reductase, Serine transhydroxy-methylase, Glycine cleavage system, Protein H, Protein P, Protein T,

35 Protein L,_Formyltetrahydrofolate dehydrogenase, Equilibrative nucleoside transporter 1, Equilibrative nucleoside transporters 2, 3, 4 & 5, Uridine phosphorylase, Thymidine phosphorylase, Orotate phosphoribosyl- transferase,

Uridine Kinase, Thymidine kinase, Deoxycytidine kinase, Ribonucleoside reductase
M1 subunit, Ribonucleoside reductase M2 subunit, Nucleoside diphosphate kinase A
subunit, Nucleoside diphosphate kinase B subunit, Uridine mono-phosphate kinase,
Deoxycytidylate kinase, Dihydropyrimidine Dehydrogenase, Dihydropyrimidinase,
5   β-ureidopropionase, Cytidine deaminase, dCMP deaminase, and Thymidylate
synthase .

116.   The method of claim 109, wherein said disease or condition is selected from
the group consisting of cancer, proliferative skin diseases, autoimmune diseases,
10   folate deficiency, cardiovascular disease, transplantation, and spina bifida .

117.   The method of claim 109, wherein said method of treatment comprises
administration of a compound effective to treat said disease or condition.

15   118.   A kit for determination of the presence or absence of at least one sequence
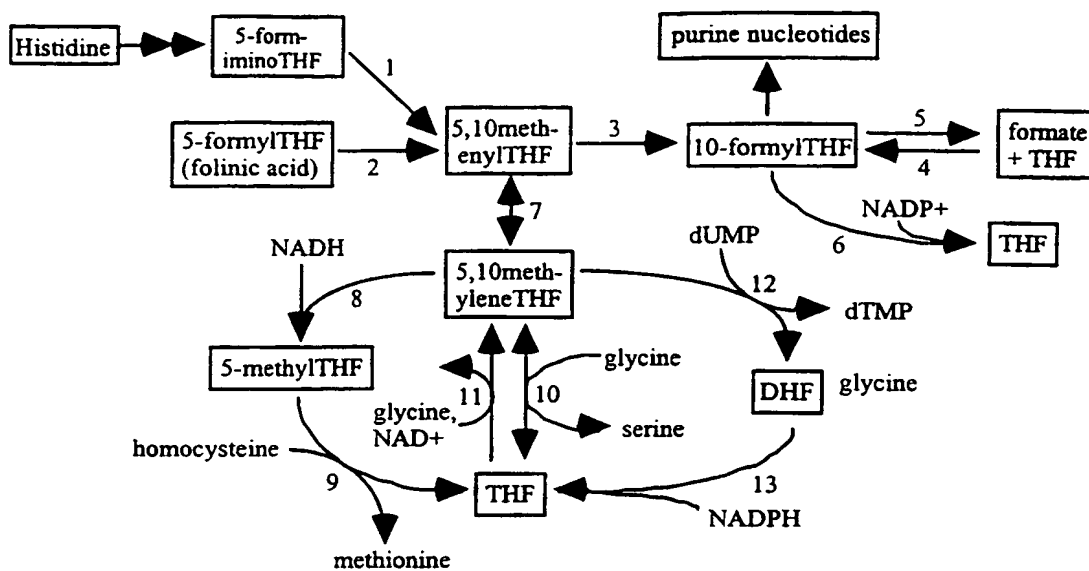variance in a gene identified in any of Tables 2, 6, and 8.

119.   The kit of claim 118, wherein said variance is listed in any of Tables 3, 4,
and 10.

Figure 1
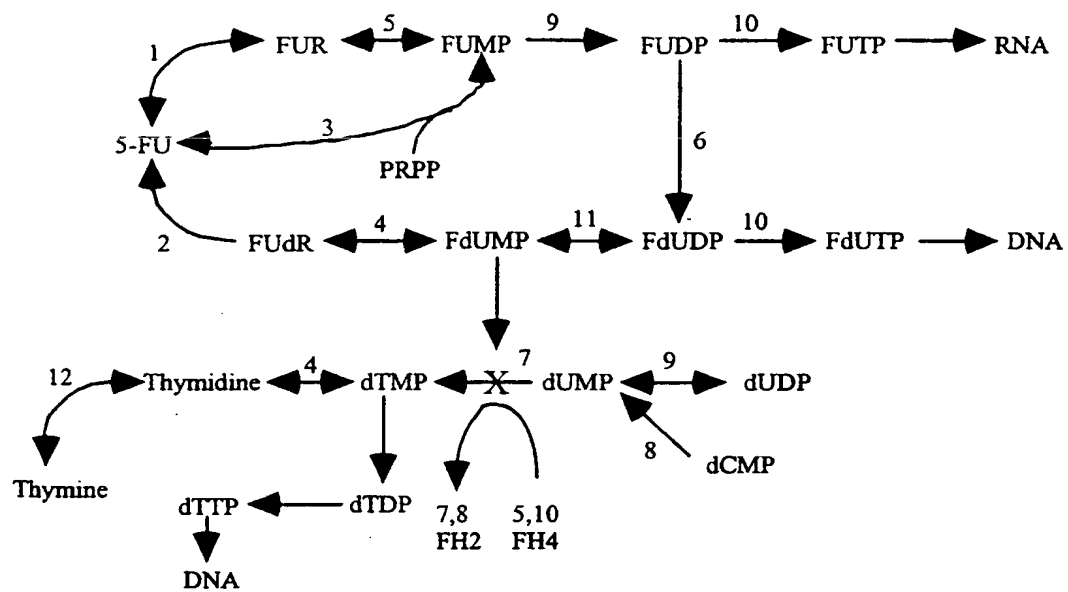
Figure 2

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MA J ET AL.: "Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer" CANCER RESEARCH, vol. 57, 1997, pages 1098-1102, XP002121822 the whole document --- -/--	1-9, 11-18, 21, 23-25, 28, 30-32, 34-50, 52, 54-59, 62-67, 71-95, 98,99, 102-106, 109-119

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 November 1999	24/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Knehr, M

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	International Application No
	PCT/US 99/16440

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document. with indication.where appropriate. of the relevant passages	Relevant to claim No.
Y	WO 98 29437 A (YOUNG JAMES D ;CASS CAROL E (CA); UNIV TORONTO (CA); BALDWIN STEPH) 9 July 1998 (1998-07-09) the whole document ---	1-9, 11-18, 21, 23-25, 28, 30-32, 34-50, 52, 54-59, 62-67, 71-95, 98,99, 102-106, 109-119
A	WO 90 02203 A (SCANLON KEVIN J) 8 March 1990 (1990-03-08) the whole document ---	
A	US 5 702 890 A (HOUSMAN DAVID E) 30 December 1997 (1997-12-30) cited in the application * see especially column 5, line 61 - column 6, line 9 * the whole document ---	
A	WO 96 40913 A (US GOVERNMENT) 19 December 1996 (1996-12-19) the whole document ---	
A	SCHALHORN A: "Therapie des forgeschrittenen kolorektalen Karzinoms" FORTSCHRITTE DER MEDIZIN, vol. 109, no. 8, 1991, pages 183-186, XP002121823 the whole document ---	
P,X	PEREIRA P ET AL.: "Loss of heterozygosity of methylenetetrahydrofolate reductase in colon carcinomas" ONCOLOGY REPORTS, vol. 6, 1999, pages 597-599, XP002121824 the whole document -----	1-119

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DOCID: <WO___0004194A1_I_>

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although claims 84-106 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects on the specied metabolic pathways and genes/enzymes.

2. [X] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

   Claims: 84,92,103,104,107,108

   see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                               [ ] No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.:   84,92,103,104,107,108

Present claims 84, 92, 103, 104, 107 and 108 relate to an unrestricted
number of possible methods, all related to methods of treating a patient
suffering from a condition or disease or methods for producing a
pharmaceutical composition/agent in general, and not revealing any link
to the specified metabolic pathways and genes/enzymes as claimed. Thus,
there is a lack of clarity allowing a complete search as well as a lack
of conciseness within the meaning of Article 6 PCT which arises to such
an extent as to render a meaningful search of the claims impossible.
Consequently, that part of the search has been directed to methods in
relation to the specified metabolic pathways and genes/enzymes as
claimed.

The applicant's attention is drawn to the fact that claims, or parts of
claims, relating to inventions in respect of which no international
search report has been established need not be the subject of an
international preliminary examination (Rule 66.1(e) PCT). The applicant
is advised that the EPO policy when acting as an International
Preliminary Examining Authority is normally not to carry out a
preliminary examination on matter which has not been searched. This is
the case irrespective of whether or not the claims are amended following
receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

nformation on patent family members

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